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ON THE NATURE OF THE EGG SURFACE DURING CLEAVAGE OF THE SEA URCHIN EGG

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INTRODUCTION

Inasmuch as the mechanism of cell division has interested many investigators, there have been a great deal of study on this problem and numerous hypotheses and theories have been put forward. Extensive reviews of them have been presented in the paper by DAN (1943) and J. C. DAN (1948). However, it may be said that there remain many unsolved problems in this field. The experiments reported in this paper are attempts to study the nature of the cortex before and during cleavage of the sea urchin egg.

Several evidences of the physiological difference between the polar and the equatorial region of the cleaving egg have already been reported by earlier workers (JUST, 1922, CHAMBERS, 1924, 1938, CHAMBERS *et al.*, 1949, MOTOMURA, 1950). Recently, MITCHISON and SWANN (1952) have shown that the change of birefringence in the cortex of cleaving eggs starts at the poles and afterwards spreads to the equator. According to SUGIYAMA's unpublished work, blister-formation takes place on the cleavage furrow of *Mespilia* eggs if they are treated with sea water of high alkalinity. These findings suggest that the egg surface in the furrow region is different in nature from that in the other regions. Since further particulars have received little investigation, it is of some importance in the study of cell division to investigate them in more detail.

MATERIALS AND METHODS

Experiments were carried out on the eggs of *Heliocidaris crassispina*, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* at the Sugashima Marine Biological Station and of *Temnopleurus toreumaticus* at the Shinmaiko Fishery Laboratory.

Eggs just before or during cleavage were put into solutions of various chemicals and the response of the cortex was observed. The chemicals used may be classified into the following four groups: haemolytic agents, protein precipitating substances, acids and detergents. Saponin, digitonin, wasp-venom and sodium choleinate belong to the first group. The second group consists of sodium tungstate, meta-phosphoric acid, and trichloracetic acid. To the third group belong hydrochloric acid and sulphuric acid. As the most suitable detergents, Monogen and Lipon were usually employed.

A series of experiments was also done to study the response of the egg

cortex to high alkalinity. In these experiments eggs were treated with NaOH or ammonia-sea water at various stages of the first cleavage.

In another series of experiments, the effect of heat was studied by exposing eggs to various temperatures ranging from 35° to 60° C. for 3 or 20 minutes.

Detailed descriptions of methods will be presented in each section of this paper.

Experiments

(*1*) *Difference of susceptibility of the cortex of the cleaving egg between the furrow and the polar region*

(*a*) *Alkali-treatments*

Eggs of several species of sea urchins were used, but the most striking effect was obtained in *Temnopleurus* eggs. Therefore, most of the experiments were performed with this species.

Eggs were treated with solutions of high alkalinity. The solutions were made up by adding 0.54 cc. of N/10 NaOH to 25 cc. of Ca-free and Mg-free sea water respectively.

When eggs were immersed in a Ca-free or Mg-free sea water of high alkalinity, blisters were formed in the region of the cleavage furrow. As shown in Fig. 1, blisters were formed within 5 minutes after the treatment at the dum-

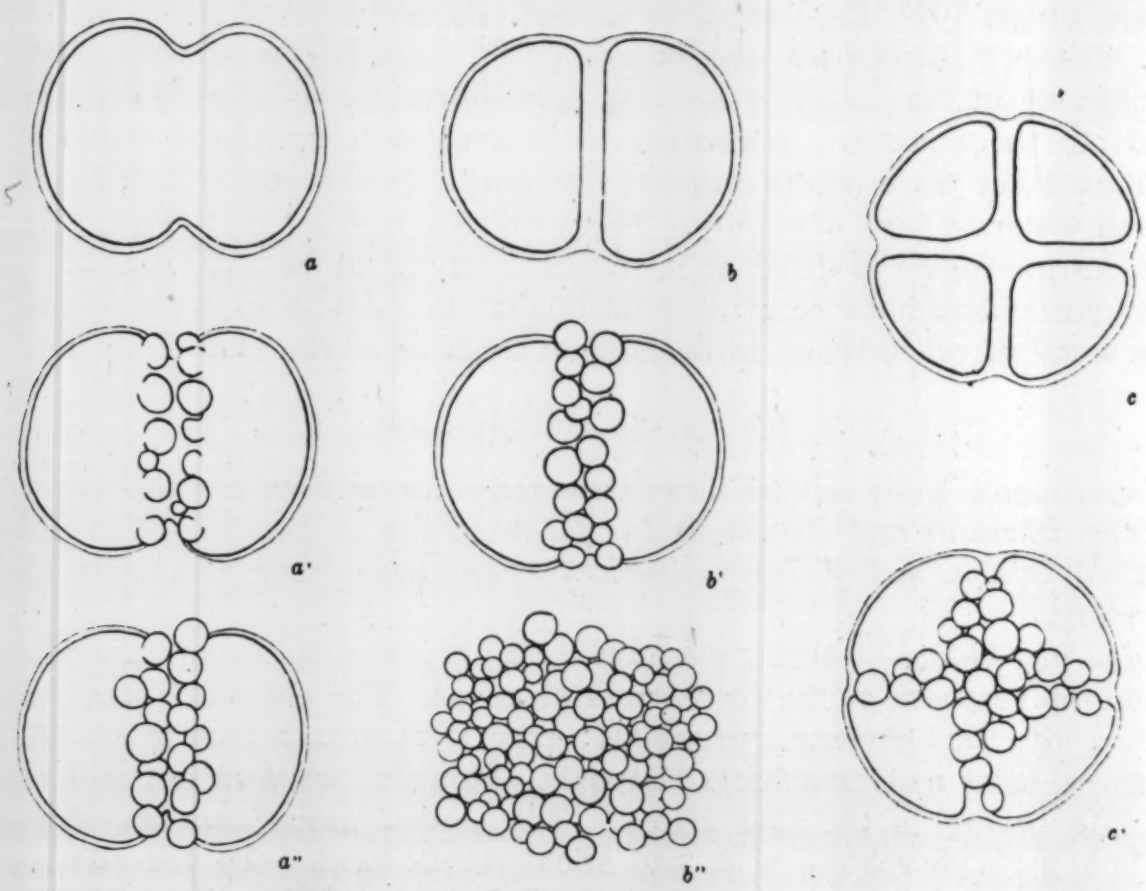

Fig. 1. Formation of blisters by alkali-treatment.

1 *a*, cleaving egg before the treatment. 1 *a'*, the same egg, a few minutes after the treatment. 1 *a''*, later than 1 *a'*.

1 *b*, cleaved egg before the treatment. 1 *b'*, the same egg, after the treatment. 1 *b''*, an egg in which blisters developed extraordinarily on the whole egg surface.

1 *c*, an egg after the second cleavage. 1 *c'*, the same egg after the treatment.

bell stage. At first, they appeared in two rows, and afterwards covered all the furrow region. When cleavage was completed, blisters were found to be covering the cleavage plane. This was also the case in the second cleavage. It was sometimes observed that blisters were formed not only in the furrow region but also on the whole egg surface, although the formation of blisters always began to take place in the furrow region. Fig. 1*b″* shows such an egg in which blisters developed to an extraordinary degree.

The reason why the blister formation occurs in the furrow region is not yet known, but it is highly probable that the furrow region is more susceptible to high alkalinity than the other regions.

(*b*) *Heat-treatment*

Temnopleurus eggs were used in these experiments. The effect of heat was tested at 35°, 40°, 45°, 50°, and 60° C. The deviation of temperature during the experiments was 0.5° C.

Eggs just before or during cleavage were put into a small glass-tube, which was filled with heated sea water at any given temperature. After treatment for 1, 3 or 20 minutes, they were transferred to a watch glass at room temperature. Observation was carried out 25 minutes after the treatment.

When eggs just before cleavage were exposed to the optimum temperature for the optimum time, the formation of blisters took place in the equatorial region. When cleaving eggs were exposed to the same condition, blisters appeared in the furrow region. At a higher temperature than the optimum, "hyalo-spines" (attachment fibres, *cf.* DAN and ONO, 1952, radial halo-layer, *cf.* MOTOMURA, 1941) appeared distinctly in the hyaline layer, the width of which was wider than normal. They were observed all around the egg surface except the furrow region or the cleavage plane.

As summarized in Table 1, blisters were produced only by the treatment at 40° C. for 1 or 3 minutes, while spines were clearly found at any temperature higher than 45° C., without relation to the treatment time.

Table 1. Formation of blisters and hyalo-spines by heat treatment

Temperature	35° C.			40° C.			45° C.			50° C.			55° C.		
Time-length of the treatment (min.)	1	3	20	1	3	20	1	3	20	1	3	20	1	3	20
Formation of blisters	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Formation of hyalo-spines	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+

Fig. 2 shows the modes of blister formation and the appearance of the hyalo-spines during the course of cleavage. When the egg is still round, just before cleavage, blisters already appear like a band in the equatorial region of the egg surface. At the dumbell stage, blisters are formed in two rows in the furrow region and after the completion of cleavage, they are found on the cleavage plane. On the other hand, the hyalo-spines, which are distributed on the whole egg surface before cleavage, are not found either in the furrow

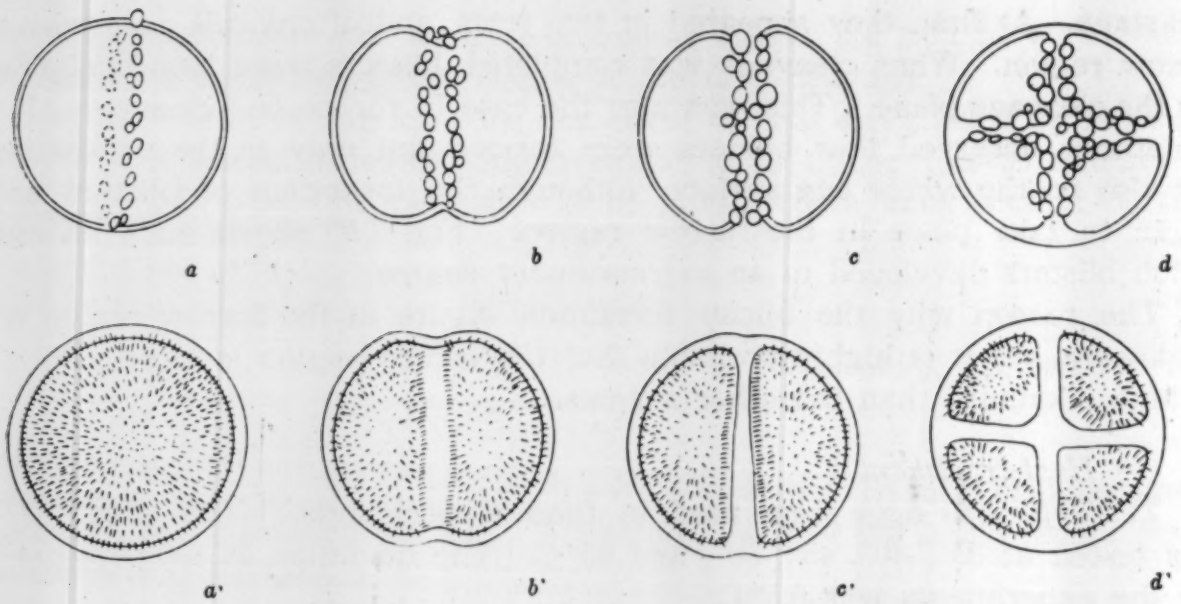

Fig. 2. Formation of blisters and hyalo-spines by heat treatment.
2 *a–d*, eggs in which formation of blisters was induced in different stages of the first and second cleavage.
2 *a'–d'*, eggs in which hyalo-spines were formed.

region of the dividing egg or on the cleavage plane at the 2-cell stage. It may be noticed that blisters are formed only on the boundary of the spine-region and the non-spine-region or in the region where the spines are not found.

(*c*) *Experiments of cytolysis*

In these experiments, eggs of *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* were used. The fertilization membrane was previously removed by the following procedure. Eggs were put into a 1 M urea solution 1 minute after insemination and were repeatedly blown and sucked by a pipette. The denuded eggs were then transferred into normal sea water.

Eggs immersed in a solution of the cytolytic reagent just before or during the first cleavage were examined to determine what region of the eggs began to cytolyze first.

Wasp-venom was obtained from the poison sac of *Polistes fadwigae*, a common species in Japan, and was used without determining the concentration. Fig. 3 shows the mode of cytolysis by means of wasp-venom. When the egg begins to elongate, the egg surface of the polar region becomes uneven and afterwards cytolysis takes place, beginning in this region. The same results are obtained in the second and third cleavages. Whether or not the egg has the fertilization membrane and the hyaline layer does not affect the results.

It was found that the effects of 0.01% digitonin, 1% sodium choleinate, 1% saponin and 1% Monogen were similar to that of wasp-venom.

When eggs were put into a 5% sodium tungstate solution, a bursting of the protoplasm always occurred in the furrow region or in the cleavage plane within a few minutes. This was especially marked in eggs which had been deprived of both the fertilization membrane and the hyaline layer (Fig. 4). The most effective pH of the solution was 8.1. It can be noted that in the egg just before elongation and cleavage, the bursting tends to take place in the equatorial region,

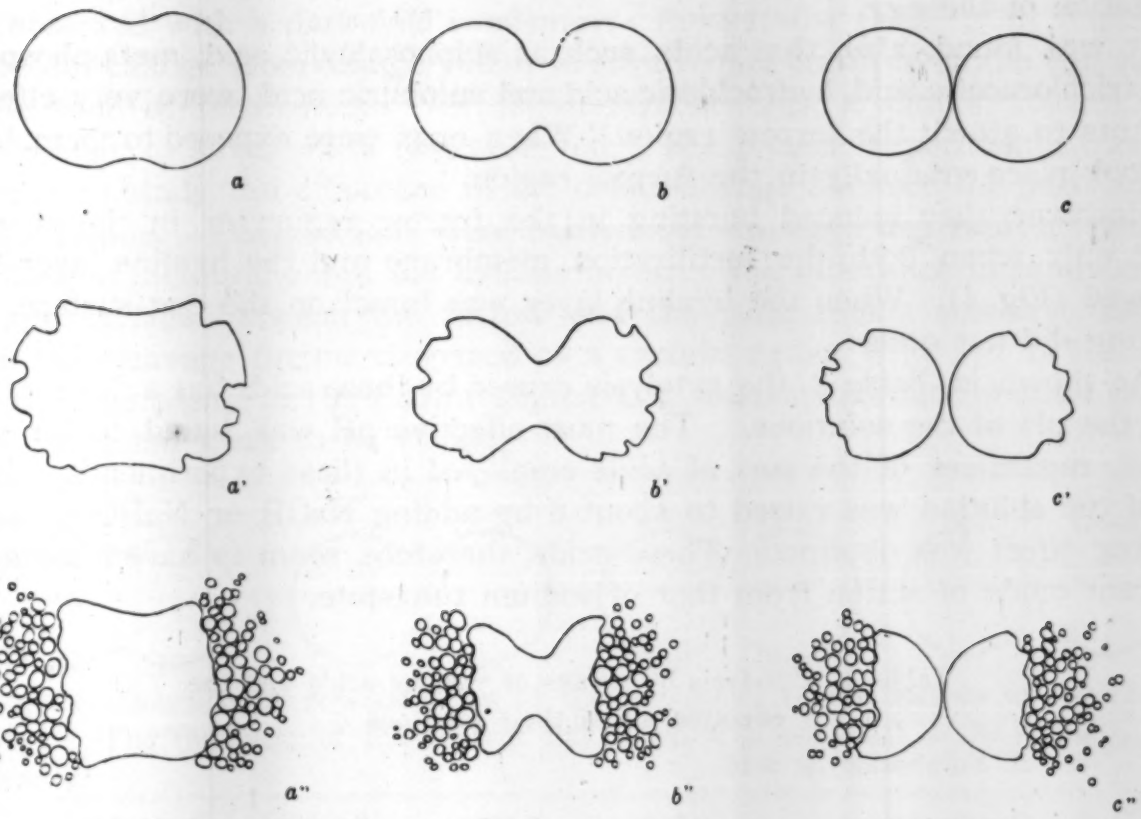

Fig. 3. Cytolysis by means of wasp-venom.
3 *a-c*, eggs deprived of the fertilization membrane, at different stages of the first cleavage.
3 *a'-c'*, the same eggs, 1-2 minutes after the treatment.
3 *a''-c''*, further change of the eggs, showing cytolysis at the polar regions.

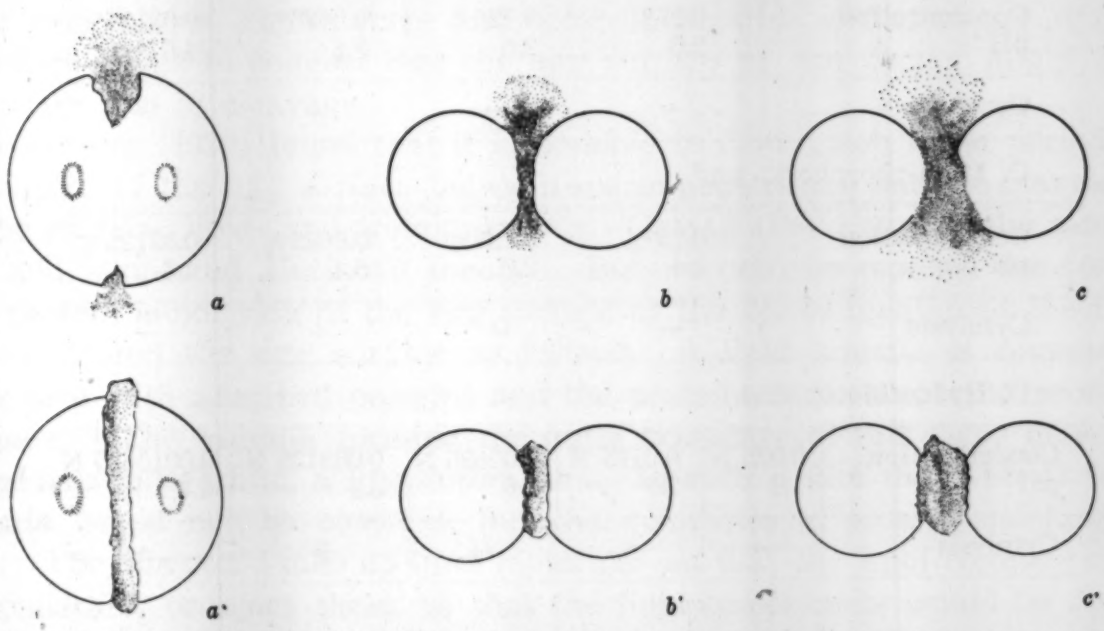

Fig. 4. Cytolysis by means of sodium tungstate solution and acid sea water solution.
4 *a-c*, eggs cytolyzed in a solution of sodium tungstate at different stages of the first cleavage.
4 *a'-c'*, eggs cytolyzed in acid sea water.

i.e. the prospective furrow region. This fact and the result obtained in the experiments of heat treatment suggest that the nature of the equatorial region may already be different from that of the polar region even at the stage before

elongation of the egg.

It was found, also, that acids, such as sulphosalcylic acid, meta-phosphoric acid, trichloracetic acid, hydrochloric acid and sulphuric acid, were very effective reagents to attack the furrow region. When eggs were exposed to them, bursting took place gradually in the furrow region.

However, they induced bursting in the furrow region or in the cleavage plane only when both the fertilization membrane and the hyaline layer were removed (Fig. 4). When the hyaline layer was intact on the egg surface, such bursting did not occur.

As shown in Table 2, the cytolysis caused by these acids has a close relation with the pH of the solutions. The most effective pH was found to be about 3.0–3.2, regardless of the sort of acids employed in these experiments. If the pH of the solution was raised to about 8 by adding NaOH or $NaHCO_3$, no cytolyzing effect was obtained. These acids, therefore, seem to have a somewhat different mode of action from that of sodium tungstate.

Table 2. Cytolysis by means of various acids and the effective pH of their solutions

A. Sulphosalcylic acid

Concentration pH	0.25% 2.2	0.125% 2.6	0.0625% 3.2	0.03125% 4.7
Cytolysis	−	+	##	−

B. Trichloracetic acid

Concentration pH	0.25% 2.0	0.125% 2.6	0.0625% 3.2	0.03125% 4.6
Cytolysis	−	−	#	−

C. Meta-phosphoric acid

Concentration pH	0.25% 2.4	0.125% 2.7	0.0625% 3.4	0.03125% 4.8
Cytolysis	−	#	+	−

D. Hydrochloric acid

Concentration pH	0.025 N 1.8	0.0125 N 2.2	0.00625 N 2.6	0 003125 N 3.2	0.0015625 N 4.4
Cytolysis	−	+	##	#	−

It is concluded that the cytolytic reagents employed in the experiments can be classified into two groups. The first group is especially effective on the polar region of the cleaving egg, inducing cytolysis first in this region. The second group attacks selectively the furrow region or the cleavage plane.

(2) Observations with a dark-field condenser

The colour-change of the egg surface in the furrow and the polar regions

was observed with a dark-field condenser. RUNNSTRÖM (1923, 1928) found that the colour-change from orange-yellow to silver-white occurred on the egg surface at the time of fertilization. In *Hemicentrotus* eggs, a similar colour-change from orange to silver-white was observed. The present experiments was done in order to study the difference in the colour-change between the polar and the furrow region. Observations were carried out on eggs deprived of both the fertilization membrane and the hyaline layer. The difference in luminosity of the egg surface between the furrow and the polar region was first observed when the cleavage furrow deepened to a certain extent (Fig. 5). It was found that the luminosity in the furrow region was weaker than that in other regions, often being too weak to be visible.

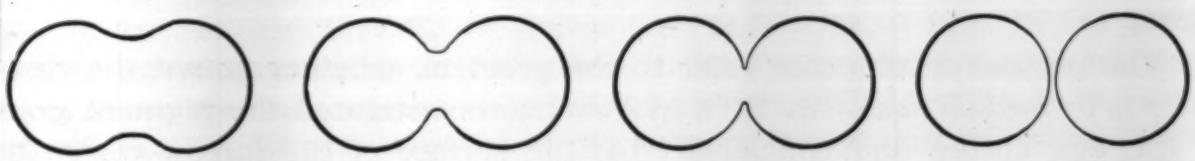

Fig. 5. Diagramatic representation of the luminosity of the surface during the first cleavage. The thickness of the contour line is made proportional to the strength of the luminosity.

DISCUSSION

It has been found that the nature of the egg surface is different between the polar and the furrow region. It is an especially interesting fact that the surface of the equatorial region is already different in nature from that of the polar region even at the stage just before elongation and cleavage of the egg. This fact seems to suggest that the egg surface or the cortex might play an important role in cleavage.

HERLANT (1920) found that it is possible to distinguish three periods as to the nature of the egg surface between sperm penetration and the first cleavage: *i.e.* the protein-, lipid-, and protein-periods. ÖHMAN (1945) studied the same problem and supported Herlant's theory. But in 1947, he reached the conclusion that protein is not rich in the egg surface at the above-mentioned third period. He considered the egg surface as follows. A lipid lamella is covered on its outer side with adsorbed proteins and the proteins form a specific protein-lipid complex. The lamella forming the outer boundary of this layer may be conceived as a mixed film, a lipoprotein film. In such a film the ratio of the components would not be constant, but the quantities of protein and lipid would vary. The film could take up lipid molecules so that the lipid component would preponderate, or eject them, so that the lipid components would be dominant. According to the opinion of ÖHMAN, such changes in the film may provide an explanation for the periodicity of the change in nature of the egg surface between fertilization and the first cleavage.

The experiments on cytolysis by means of various chemicals furnish an experimental basis for the view that protein may be exposed more abundantly than lipid in the surface of the cleavage furrow and the cleavage plane. It has been found that cytolysis starts at the polar region when it is caused by

wasp-venom, which contains lecithase and acts on lecithin, and by Monogen and digitonin, which easily form molecular compounds with cholesterin. It has also been found that eggs are attacked readily in the furrow and the cleavage plane by protein precipitating substances and acids such as sodium tungstate and sulphosalcylic acid. These findings and ÖHMAN's conclusion suggest that the protein components are dominant and lipid components are sparse in the furrow region and the cleavage plane.

FREY-WYSSLING says that in the so-called protein phase, proteins are in a more solid, gel-like state and the "Hauptpunkten" between the protein chains are numerous. If one presumes that the egg surface of the furrow region is of protein nature, gelation might take place there. This conception may be related to MARSLAND's idea, that cleavage occurs by gelation in the furrow region.

The present results also refer to the problem, whether or not the cleavage plane is formed *de novo*. In 1936, MOTOMURA investigated the pigment granules in the egg cortex and concluded that the surface of the furrow region might be newly formed, because the granules could not be detected in that region. This observation seems to coincide with the author's result on the hyalo-spine formation by heat treatment. However, MOTOMURA noted in his recent work (1950) that the formation of a new membrane took place after the constriction in the furrow region was completed. It seems necessary to await much more information before we can form a consistent picture of the formation of the cleavage plane.

The author is glad to express his hearty gratitude to Dr. M. SUGIYAMA under whose direction the present experiments were undertaken and to Mr. M. ISHIKAWA and Mr. E. NAKANO for their kind advice and offer of many chemicals. The thanks are also due to Dr. T. YAMAMOTO who kindly taught the method of taking out wasp-venom and to Dr. K. DAN and Dr. J. C. DAN for their correction of the manuscript.

SUMMARY

(1) Responses of the surface of the cleaving egg to various agents were studied.

(2) Materials used were *Pseudocentrotus depressus*, *Heliocidaris crassispina*, *Hemicentrotus pulcherrimus* and *Temnopleurus toreumaticus*.

(3) When cleaving eggs are immersed in a Ca-free or Mg-free sea water of high alkalinity, blisters are formed in the region of the cleavage furrow.

(4) When eggs just before cleavage are exposed to 40° C., the formation of blisters takes place in the equatorial region. When cleaving eggs are exposed to the same condition, blisters appear in the furrow region. At higher temperature, the hyalo-spines are formed all around the egg surface except the furrow region.

(5) Several haemolytic agents and detergents have been found to induce cytolysis of the cleaving egg, usually beginning at the polar region.

(6) Protein precipitating substances and certain acids have been found to attack the furrow region of the cleaving egg selectively.

(7) The luminosity of the egg surface was observed with a dark-field condenser. The luminosity in the furrow region is weaker than that in the other regions.

(8) Some discussions were given, based on these experiments.

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COMPARATIVE STUDIES ON EXPERIMENTAL FORMATION OF MULTINUCLEATED EGGS OF SEA URCHINS BY MEANS OF VARIOUS AGENTS

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It is a well-known fact that nuclear division is usually accompanied by cell division or cleavage. But under certain conditions the former takes place without the latter, and consequently so-called syncytia are formed.

Since the early work of O. and R. HERTWIG (1887), numerous agents have been found to be effective for inducing the formation of syncytia in eggs of sea urchins. WILSON (1901) and POLOWZOW (1924), for instance, found that nuclear division without cytoplasmic division took place under the influence of narcotics such as ether or alcohol.

Recently, the author found that surface active substances, such as detergents, have similar effects. Studying comparatively the formation of multinucleated eggs by means of narcotics and of surface active substances, it was found that there are some differences in the mode of action between these two groups of reagents.

Since these findings seem to pose certain interesting problems as to the mechanism of cell division, they will be presented here.

MATERIAL AND METHODS

The experiments were conducted on the eggs of four sea urchins, *Heliocidaris crassispina, Pseudocentrotus depressus, Hemicentrotus pulcherrimus* at the Sugashima Marine Biological Station, and *Temnopleurus toreumaticus* at the Shinmaiko Fishery Laboratory.

In the first series of experiments, detergents, such as Monogen and Lipon, and haemolytic reagents, such as saponin, digitonin and sodium choleinate, were used. It was found that Monogen was the most effective reagent for the purpose. A 1 per cent solution of Monogen was made with sea water as a stock solution. After its filtration, solutions of graded concentrations were made by a method of successive half-dilutions with sea water. Solutions of other reagents were prepared in the same way.

Urethane, ethyl alcohol and colchicine were used in another series of experiments in order to compare their effects with that of the surface active substance. A 1 M solution of urethane in distilled water, a 10% solution of ethyl

alcohol in sea water and an 0.0008 M solution of colchicine in sea water were made as stock solutions, respectively. For the experiments they were diluted in the same way as the Monogen solution.

Fertilized eggs were put into solutions of the reagent to be tested 5 minutes after fertilization or just before the first cleavage. Eggs deprived of the fertilization membranes were sometimes used.

In a series of experiments, a dark-field condenser was used to study the colour-change of the egg surface. In order to observe it fully, the following procedure was employed. A small amount of vaseline was put on the four corners of a cover-glass and this was placed over the egg sea water drop on a slide. The eggs were adequately pressed down by pushing the cover-glass with a fine needle to prevent shifting of the eggs during the subsequent procedure. Several drops of the solution to be tested was placed on the slide at one side of the cover-glass. A small piece of filter paper was applied to the other side of the cover-glass so that the solution ran into the space beneath the cover-glass. In this condition, changes of colour of the egg surface were observed with a dark-field condenser.

In order to determine the protoplasmic viscosity of eggs which were immersed in one of the solutions, the centrifuging method was adopted.

Experimental Results

(1) *Formation of multinucleated eggs following treatment with a surface active substance*

It was found that when fertilized eggs of sea urchins were immersed in an appropriate solution of the surface active substance, their cytoplasmic divisions were suppressed while their nuclear divisions went on normally, so that multinucleated eggs were formed. Reagents used for these experiments were Monogen, Lipon, saponin, digitonin and Na-choleinate.. All the reagents were found to be effective, but the most striking effect was obtained with Monogen in *Hemicentrotus* eggs.

Table 1 summarizes the results of the experiments with Monogen, presenting percentages of multinucleated eggs formed under various conditions. It is shown that the results are much the same without reference to the time of immersion

Table 1. Formation of multinucleated eggs by means of Monogen
(Room temp. 18° C.)

Time of immersion	5 min. after fertilization			1 hr. 22 min. after fertilization		
Conc. of Monogen (%)	Eggs cytolyzed (%)	Multinucleated eggs, failing to cleave (%)	Intact eggs (%)	Eggs cytolyzed (%)	Multinucleated eggs, failing to cleave (%)	Intact eggs (%)
1/2	83	17	0	98	2	0
1/4	72	28	0	25	75	0
1/8	23	77	0	15	85	0
1/16	0	100	0	0	100	0
1/32	0	24	76	0	28	72
1/64	0	0	100	0	0	100

of the eggs. It is also shown that the most suitable concentration of Monogen for the formation of multinucleated eggs is 1/16%. With this concentration, there is not even any retardation of the nuclear division of the treated eggs, although the cytoplasmic division is completely suppressed. When a higher concentration of Monogen is used, the eggs are sometimes partially cytolyzed. However, these eggs also become multinucleated.

The hyaline layer of the multinucleated eggs is thicker than that of the controls. However, since the eggs deprived of the hyaline layer also become multinucleated following treatment with Monogen, it is not conceivable that the change in the hyaline layer is the cause of the suppression of the cytoplasmic division.

(2) *Comparative studies on the formation of multinulceated eggs by means of various reagents*

(*A*) As described in the previous section, it was found that a surface active substance such as Monogen can induce the formation of multinucleated eggs. On the other hand, numerous reagents, such as urethane and ethyl alcohol, have been known to have a similar effect. Since these reagents are different from each other in nature, it is interesting to study comparatively their mode of action.

Fertilized eggs were put into a solution of urethane or ethyl alcohol 5 minutes after fertilization. It was ascertained that these reagents induce the formation of multinucleated egg if their concentrations were appropriate. As shown in Tables 2 and 3, the most effective concentrations of urethane and ethyl alcohol were 1/16 M and 2.5% respectively. It should be noted that all the multinucleated eggs thus formed remained undivided, as in the case of Monogen.

In the next experiment, fertilized eggs were transferred into the solution just before the cleavage furrow began to be formed. If the eggs were im-

Table 2. Formation of multinucleated eggs by means of urethane
(Room temp. 18° C.)

Time of immersion	5 min. after fertilization			1 hr. 22 min. after fertilization				
	Eggs which failed to cleave from the first cleavage			Eggs which failed to cleave from the first cleavage		Eggs which failed to cleave from fhe 2-cell stage		
Conc. of unethane (M)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Intact eggs (%)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Intact eggs (%)
1/2	100	0	0	100	0	0	0	0
1/4	100	0	0	100	0	0	0	0
1/8	100	0	0	63	0	37	0	0
1/16	0	100	0	0	8	0	92	0
1/32	0	14	86	0	0	0	0	100
1/64	0	0	100	0	0	0	0	100

Table 3. Formation of multinucleated eggs by means of ethyl alcohol (Room temp. 18° C.)

Time of immersion	5 min. after fertilization			1 hr. 22 min. after fertilization				
	Eggs which failed to cleave from the first cleavage			Eggs which failed to cleave from the first cleavage		Eggs which failed to cleave from the 2-cell stage		
Conc. of ethyl alcohol (%)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Intact eggs (%)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Eggs, in which both nuclear and cytoplasmic division were impaired (%)	Multinucleated eggs (%)	Intact eggs (%)
10	100	0	0	100	0	0	0	0
5	100	0	0	75	0	25	0	0
2.5	0	100	0	0	10	0	90	0
1.25	0	6	94	0	0	0	0	100
0.625	0	0	100	0	0	0	0	100

mersed in 1/16 M urethane or 2.5% ethyl alcohol, most of them become multinucleated but in this case the first cleavage was not inhibited, resulting in two cells, each of which became multinucleated afterwards. In the experiments with Monogen, on the contrary, the first cleavage was suppressed even if eggs were put into the solution just before cleavage. The same result was obtained in eggs deprived of the fertilization membrane.

The effect of colchicine, also, was studied by a similar method. As shown in Table 4, its effect is not necessarily similar to that of the narcotics.

Table 4. Inhibition of cleavage by means of colchicine (Room temp. 18° C.)

Time of immersion	5 min. after fertilization		1 hr. 22 min. after fertilization		
Conc. of colchicine (M)	Eggs which failed to cleave from the first cleavage (%)	Intact eggs (%)	Eggs which failed to cleave from the first cleavage (%)	Eggs which failed to cleave from the 2-cell stage (%)	Intact eggs (%)
0.0008	100	0	5	95	0
0.0004	100	0	7	93	0
0.0002	100	0	5	95	0
0.0001	100	0	0	0	100
0.00005	88	12	0	0	100
0.000025	0	100	0	0	100

These facts suggest that there are differences in the mode of action between surface active substances and narcotics. It seems probable that the former may attack the egg surface quickly so that even the first cleavage does not occur, while the latter may act more or less slowly and consequently the first cleavage is not suppressed, but the subsequent cleavages are inhibited.

(*B*) *Comparative observations by means of dark-field condenser*

The egg surface was observed by means of a dark-field condenser in order

to study whether or not a difference in luminosity can be found between multinucleated eggs resulting from Monogen treatment and those induced by urethane, ethyl alcohol or colchicine. In these experiments, the eggs were deprived of both the fertilization membrane and the hyaline layer, in order to permit accurate observation of the egg surface. The surface of multinucleated eggs, treated with Monogen, was found to shine with an extremely brilliant reddish-orange colour, while those treated with urethane, ethyl alcohol or colchicine showed a silver-white luminosity, similar to that of the control eggs.

More detailed observations were performed by the procedure described before, in order to determine when the colour-change of the egg surface occurred in a Monogen solution. It was found that, within 2–3 minutes after the eggs were exposed to a 1/16% Monogen solution, the luminosity of the egg surface increased gradually.

After 5-6 minutes the egg surface became rather luminous and its colour changed from silver-white to reddish-orange. After 7-8 minutes, the egg surface shone in brilliant reddish-orange and the intensity of the luminosity reached the maximum.

When a lower concentration (1/32%) of Monogen was used, some eggs showed a brilliant luminosity while the rest remained unchanged, shining in silver-white. It was found that the former became multinucleated eggs afterwards, while the latter cleaved normally.

From these observations, it was concluded that the colour-change of the egg surface is induced by Monogen but not by the narcotic, and that this change caused by Monogen become visible in a short time after the treatment.

(C) Comparative studies on the viscosity of the multinucleated eggs

These experiments were performed with the eggs of *Heliocidaris crassispina*, since the eggs of this species are easy to stratify by centrifugal force.

Eggs were put into solutions of the reagents described above, 5 minutes after fertilization, and multinucleated eggs were formed. When the control eggs were at the 8- or 16-cell stage about 2.5 hours after fertilization, these multinucleated eggs were centrifuged at 1,000 × g for 3–4 minutes. The eggs treated with urethane, ethyl alcohol or colchicine were clearly stratified by this procedure, but those treated with Monogen were quite unaffected, being much the same as the control eggs. A similar result was obtained when the centrifugation was performed one hour after fertilization.

It is of interest to note that eggs are stratified by a similar centrifugal force even when treated with a lower concentration of narcotic or colchicine. When eggs were immersed in a solution of urethane, ethyl alcohol or colchicine of a lower concentration, their cleavage were usually not suppressed, so that no multinucleated eggs were formed. However, the stratification was easily brought about by the centrifugation. This fact means that cleavage may be able to take place normally even if the viscosity of the protoplasm decreases to a certain extent.

In the next experiments, unfertilized eggs were immersed in the solution in question for one hour, and were centrifuged in the same way as described above. In this case, the eggs treated with Monogen were also found to differ

from those treated with other agents, the former being unaffected while the latter were distinctly stratified.

It is concluded that the viscosity of the cytoplasm decreases in both fertilized and unfertilized eggs following the treatment with urethane, ethyl alcohol or colchicine, but Monogen has no effect of this kind.

Discussion

It has been found, as stated above, that surface active substances, such as Monogen, inhibit the cleavage of sea urchin eggs without impairing the nuclear division and consequently induce the formation of multinucleated eggs. Narcotics, such as urethane and alcohol, also show a similar result. But their effects on the eggs are somewhat different in nature from those of the surface active substance. Monogen inhibits the cleavage almost immediately after the eggs are exposed to it, but urethane attacks the eggs more slowly. In the observation with a dark-field condenser, the surface of the egg in Monogen shows a change in colour from silver-white to reddish-orange, while the egg treated with urethane or alcohol remains unchanged. The centrifuging experiments show that Monogen has no effect on the viscosity of the protoplasm of the egg, while a decrease of viscosity is observed in narcotized eggs.

On the basis of these facts, it may be postulated that Monogen impairs the egg cortex, while the narcotic rather affects the inner protolpasm. If this postulation be correct, it may be said that the cytoplasmic division is inhibited when the egg cortex has been impaired. On the other hand, the suppression of aster-formation by narcotics also results in the failure of cytoplasmic division. It is, therefore, highly probable that both the egg cortex and aster play important roles in the mechanism of the cytoplasmic division.

Beams and Evans (1940) investigated the effect of colchicine on the cleavage in *Arbacia punctulata*. According to them, colchicine inhibits cleavage if applied sooner than appraximately 22 minutes after fertilization, but cleavage takes place if the eggs are allowed to develop 28 to 36 minutes before being placed in the colchicine. They state that the abnormally rapid disintegration of the aster in the eggs, once they have reached the late anaphase stage, has little or no effect on the cleavage process, and that the chief action of the colchicine is to lower the viscosity or to inhibit and destroy the increased gelation that normally occurs within the eggs during division.

These results coincide with those of the author, indicating that colchicine disturbs the nuclear division. Therefore, the mechanism by which colchicine inhibits cytoplasmic division is rather similar to the way in which narcotics have been found to act.

The author is glad to acknowledge his indebtedness to Dr. M. Sugiyama for his invaluable instruction throughout the course of this work and to Dr. K. Dan and Dr. J. C. Dan for their correction of the manuscript.

Summary

(1) Eggs of the sea urchins, *Hemicentrotus pulcherrimus, Pseudocentrotus*

depressus, *Heliociadaris crassispina* and *Temnopleurus toreumaticus* were used as material.

(2) The formation of multinucleated eggs was induced by surface active substances. The most striking effect was obtained with Monogen.

(3) There are differences in the mode of action of surface active substances and narcotics.

(4) Monogen inhibits the cleavage almost immediately after the eggs are exposed to it, but urethane impairs the eggs more slowly.

(5) Observation with a dark-field condenser shows that a colour-change in the egg surface is induced by Monogen, but not by the narcotic.

(6) The viscosity of the cytoplasm decreases in both fertilized and unfertilized eggs following treatment with urethane, ethyl alcohol or colchicine, but Monogen has no effect of this kind.

(7) It was postulated that Monogen impairs the egg cortex, while the narcotic and colchicine rather affect the inner protoplasm. It may be said that the cytoplasmic division is inhibited when the egg cortex has been impaired.

Literature Cited

BEAMS, H. W. and T. C. EVANS, 1940. Some effects of colchicine upon the first cleavage in *Arbacia punctulata*, Biol. Bull., *79*, 188-198. — HERTWIG, O. und R., 1887. Über den Befruchtungs- und Theilungsvorgang des thierischen Eies unter dem Einfluss äusseren Agentien, Jena. Ztsch., *20*, 120-141. — POLOWZOW, E., 1924. Über die Wirkung der Alkoholnarkose auf die Entwicklung der Seeigel-eier II. Polysyncytien, Arch. f. mikr. Anat. u. Entw. Mech., *103*, 1-67. — WILSON, E. B., 1901. Experimental studies in cytology. II. Some phenomena of fertilization and cell division in etherized eggs. III. The effect on cleavage of artificial obliteration of the first cleavage furrow. Arch. f. Entw. Mech., *13*, 353-395.

THE ACTIVATION OF SEA URCHIN EGGS BY MEANS OF PHENOLS

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In the course of studies on the physiological effects of various simple organic compounds on the unfertilized egg of the sea urchin, it has been found that the egg can be activated by means of phenols, so that the fertilization membrane is formed. Since various activating reagents are considered to be useful for analyzing the mechanism of activation, the results are presented here somewhat in detail.

Materials and Methods

The eggs of the following sea urchins were employed; *Hemicentrotus pulcherrimus, Heliocidaris crassispina, Pseudocentrotus depressus* and *Temnopleurus toreumaticus.* As stimulating reagents, the following organic compounds were tested; 1) aromatic carbohydrates (benzene, toluene, xylene), 2) aniline derivatives (aniline, toluidine, *p*-aminobenzoic acid methylester, *p*-aminobenzoic acid ethylester, benzanilide, diethylaniline, dimethylaminobenzaldehyde, diphenylamine, benzidine, acetanilide), 3) phenol derivatives (phenol, resorcin, hydroquinon, pyrogallol, cresol, thymol, vanillin), 4) naphthalene derivatives (naphthalene, naphthol, naphthylamine, "Yatren"), 5) purine derivatives (caffeine, uric acid), 6) pyrrol derivatives (indole, scatole, isatine, carbazole).

In the first series of experiments, the effects on unfertilized eggs of the above reagents were studied. Ripe unfertilized eggs were exposed to the reagent dissolved in sea water, and their responses were observed before and after washing with normal sea water. The pH of the employed solution was, if necessary, adjusted to that of normal sea water (pH 8.2).

Another series of experiment was performed to test whether thymol, which was found to be effective in inducing membrane formation, could also induce cleavages and further development of the egg. In these experiments, Loeb's method of double treatment was adopted. All possible precautions were taken in order to avoid accidental sperm contamination.

Experimental Results

A. Effects of various organic compounds on unfertilized eggs

First, the experiments with thymol will be reported. Thymol solutions of various concentrations were prepared as follows: to each 10, 5, 4, 3, 2 and 1 cc.

of saturated thymol in sea water were added respectively 0, 5, 6, 7, 8 and 9 cc. of sea water. Exposing the unfertilized eggs of *Hemicentrotus pulcherrimus* to them, the effects of thymol were studied.

Under the influence of thymol of higher concentrations than a mixture of 5 cc. of saturated thymol plus 5 cc. of sea water, membranes were almost instantaneously elevated and then cytolysis followed. When unfertilized eggs were immersed in a mixture of 3 cc. of saturated thymol plus 7 cc. of sea water for 30 seconds and then removed to normal sea water, the highest percentage of normal membrane elevation was obtained. The cortical granules of these eggs had broken down and the elevated membrane seemed to be quite normal. No membrane elevation occurred when unfertilized eggs were exposed to thymol solutions weaker than a mixture of 2 cc. of saturated thymol plus 8 cc. of sea water.

Similar results were obtained with the eggs of *Temnopleurus toreumaticus*, *Pseudocentrotus depressus* and *Heliocidaris crassispina*.

The effects of the other chemicals listed above were also examined by the same procedure as that of thymol. Saturated solutions of these substances were usually used as stock solutions, since most of the chemicals were difficult to

Table 1. Effectiveness of various organic compounds in inducing membrane formation in unfertilized eggs of *Hemicentrotus pulcherrimus*

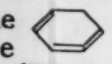
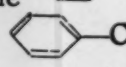
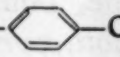
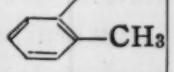
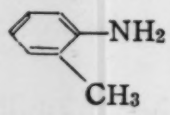
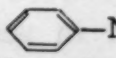
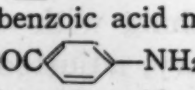
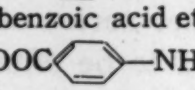
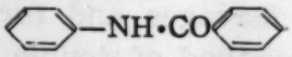
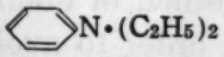
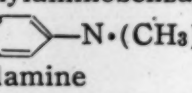
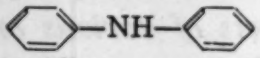
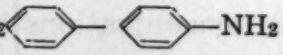
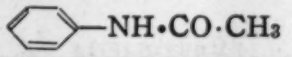
Effective chemicals	Optimum concentration of the effective chemical	Non-effective chemicals
1) (Aromatic carbohydrates)		
*Benzene 	Saturated in sea water	
*Toluene 	〃	*p*-Nitrotoluene NO_2—  —CH_3
*Xylene (*o*-, *m*-, *p*-,) 	〃	
2) (Aniline derivatives)		
Toluidine (*o*-, *p*-,) 	5 cc. of sat. toluidine plus 1 cc. of sea water	Aniline 
		p-Aminobenzoic acid methylester 
		p-Aminobenzoic acid ethylester 
		Benzanilide 
		Diethylaniline 
		p-Dimethylaminobenzaldehyde 
		Diphenylamine 
		Benzidine 
		Acetanilide 

Table 1. (*Continued*)

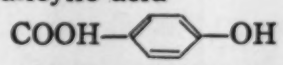
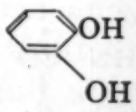
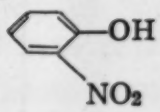
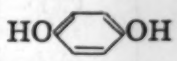
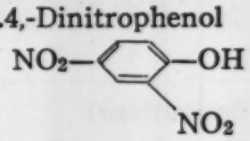
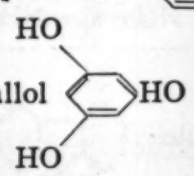
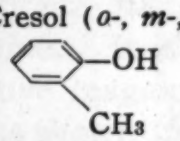
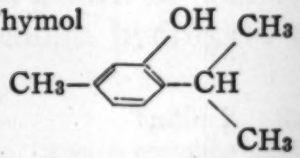
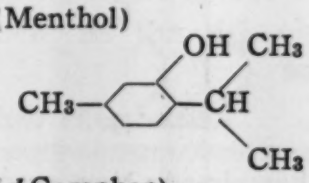
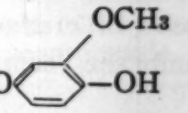
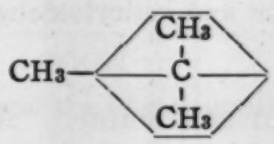
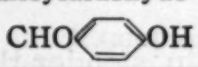
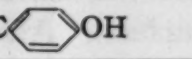
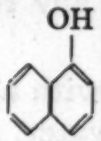
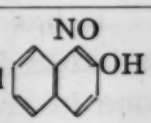
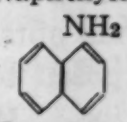
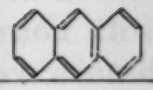
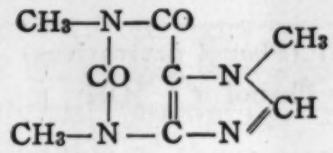
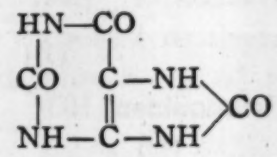
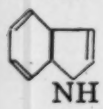
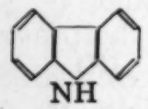
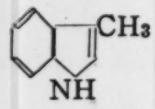
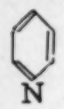
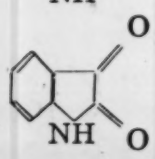
Effective chemicals	Optimum concentration of the effective chemical	Non-effective chemicals
3) (Phenol derivatives)		
Phenol 	0.3%	Salicylic acid 
Resorcin 	2.5%	*o*-Nitrophenol 
Hydroquinon 	Saturated in sea water (about 5.9%)	2.4,-Dinitrophenol 
Pyrogallol 	12%	
Cresol (*o*-, *m*-, *p*-,) 	2 cc. of sat. cresol plus 8 cc. of sea water.	
Thymol 	3 cc. of sat. thymol plus 7 cc. of sea water	(Menthol) 
Vanillin 	0.2%	(*d*-Camphor) 
(Benzaldehyde) 	Saturated in sea water	
*Salicylaldehyde 		
		p-Hydroxybenzoic acid butylester 
4) (Naphthalene derivatives)		
Naphthol (α-, β-) 	2 cc. of sat. naphthol plus 8 cc. of sea water	α-Nitroso-β-naphthol 
		Napthalene 
		Naphthylamine (α, β) 
		Yatren
		Anthracene 

Table 1. (*Continued*)

Effective chemicals	Optimum concentration of the effective chemical	Non-effective chemicals
5) (Purine derivatives)		Caffeine  Uric acid 
6) (Pyrrol derivatives)		
Indole 	Saturated in sea water	Carbazole 
Scatole 	〃	(Pyridine) 
Isatine 	〃	

* It has already been reported by Herbst (1893) and Loeb (1913) that benzene, tolene, xylene and salicylaldehyde are effective in causing membrane formation.

dissolve in sea water. At the time of use, they were diluted with sea water. The results of these experiments are summarized in Table 1.

When unfertilized eggs were exposed to *p*-cresol or vanillin, slender threads or strands were sometimes observed extending between the membrane and the egg surface. Afterwards these strands gradually broke and contracted.

It has been known that the membrane formation in the sea urchin egg can be induced by various lipid-solvents. Therefore, some experiments have been done in order to determine whether the effect of thymol has any relation with lipid.

Lipid was extracted from unfertilized *Hemicentrotus* eggs with alcohol-ether. The lipid residue thus obtained was dissolved in thymol-sea water, and unfertilized eggs were exposed to it. It was found that no membrane formation occurred in this solution. If these treated eggs were inseminated after washing with normal sea water, fertilization took place, and normal fertilization membranes were formed. This means that the thymol solution exerts no influence on the egg when lipid has been added to the solution. It is, therefore, very probable that the effect of thymol has a close connection with the lipid in the cortex of the egg.

B. Artificial parthenogenesis by means of phenols

The question arises as to whether eggs treated with phenols can develop parthenogenetically to plutei. The next experiments were therefore performed, using thymol as an activating agent. The method of the double treatment of LOEB was adopted. Unfertilized eggs of *Pseudocentrotus depressus* were treated with a mixture of 3 cc. of saturated thymol in sea water plus 7 cc. of sea water for 50 seconds and then removed to sea water. After 20 minutes, they were immersed in hypertonic sea water (8 cc. of 2.5 M NaCl plus 50 cc. of sea water) for 50 minutes and again returned to sea water. Development of the eggs started in a short time and top-swimming plutei were obtained in a few days.

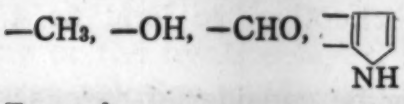
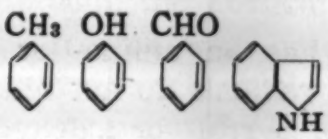
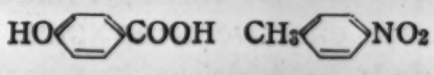
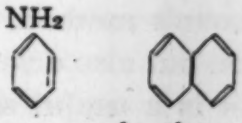
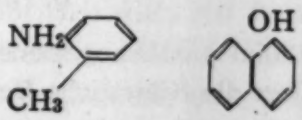
This result shows that thymol can induce not only membrane formation, but also development of the egg. Thus it is obvious that thymol is a useful activating reagent.

DISCUSSION

It has been found that various phenols are effective for the induction of membrane formation in sea urchin eggs. However, not all the phenols are effective. It is of interest that some compounds, closely related to a certain effective reagent, are often found to be of no effect.

As shown in Table 1, compounds, in which a benzene nucleus has a methyl-, a phenolic hydroxyl-, a pyrrol- or an aldehyde-radical as the side-chain, are

Table 2. Relation between side chains and their effectiveness

Side-chain in effective compounds	Side-chain in ineffective compounds
$-CH_3$, $-OH$, $-CHO$,  Examples; 	$-NO_2$, $-COOH$ (These seem to cancel the effectiveness.) Examples; 
	$-NH_2$,  Examples;  (In these compounds, the radicals cancel the effectiveness of the benzene.)  (The radicals do not change the effectiveness, when the compounds have a methyl-, a hydroxyl-, a pyrrol- or an aldehyde-radical.)

effective, so long as nitro- or carboxyl-radicals are not present. Since such compounds are generally more effective than benzene, these radicals seem to play a significant role in the action of the compounds. The nitro- and carboxyl-radicals may be considered to cancel this effectiveness. Compounds in which a benzene nulceus has an amino- or a benzene-radical as the side-chain, show no effectiveness, but these radicals do not change the effectiveness of compounds which have a methyl-, a phenolic hydroxyl-, a pyrrol- or an aldehyde-radical.

The effectiveness of the various side chains is summarized in Table 2.

It has been shown, also, that thymol and naphthol are effective reagents, but menthol, in which the bezene-nucleus of thymol is saturated, and naphthalene are of no effect. On the basis of these facts, it seems probable that in these compounds, the phenolic hydroxyl-radical plays a leading role in the induction of the membrane formation.

The author wishes to express his sincere thanks to Dr. M. SUGIYAMA for his kind criticism and valuable advice. Thanks are also due to Dr. K. DAN and Dr. J. C. DAN for their correction of the manuscript, and to Dr. Y. TAKATORI for the generous supply of the chemicals.

SUMMARY

1. The effects of phenols on unfertilized eggs of sea urchins were studied. *Hemicentrotus pulcherrimus, Heliocidaris crassispina, Pseudocentrotus depressus* and *Temnopleurus toreumaticus* were used as material.

2. It was found that various phenols are effective in inducing membrane formation.

3. Compounds in which a benzene-nucleus has a methyl-, a phenolic hydroxyl-, a pyrrol- or an aldehyde-radical, are effective, so long as a nitro- or carboxyl-radical is not present.

4. The nitro- and the carboxyl-radical may be considered to cancel the effectiveness of the effective compounds.

5. Compounds in which a benzene-nucleus has an amino- or a benzene-radical, show no effectiveness. However, these radicals do not reduce the effectiveness when a methyl-, phenolic hydroxyl-, pyrrol- or aldehyde-radical is present as a side-chain.

6. Employing LOEB's method, it was found that thymol can induce not only membrane formation but also development of the egg. Therefore, it may safely be said that thymol is a useful activating reagent.

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RELATION BETWEEN THE BREAKDOWN OF THE CORTICAL GRANULES AND PERMEABILITY TO WATER IN THE SEA URCHIN EGG

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R. S. LILLIE (1916, 1917) demonstrated that after fertilization, *Arbacia* eggs take up water by osmosis several times more rapidly than before fertilization. This finding has been supported by observations of a number of authors (MCCUTCHEON and LUCKÉ, 1926, NORTHROP, 1928, MCCUTCHEON, LUCKÉ and HARTLINE, 1931 *a*, *b*, HOBSON, 1932). Furthermore, STEWART (1931) showed an increase in permeability to a non-electrolyte such as ethylene glycol. ABELSON's work (1947), with labeled phosphate, also indicated an increased ion uptake after fertilization.

One of the most remarkable morphological changes in the cortex at the time of fertilization is the breakdown of the cortical granules. A question arises as to the possibility that the granular breakdown has some relation to the change of permeability.

MOORE (1930) and MOTOMURA (1934) have found that the egg of the sea urchin can be fertilized and caused to develop without either the fertilization membrane or the hyaline layer, if the egg be previously treated with butyric acid or urea solution. MOTOMURA (1941) has found, also, that in eggs treated with butyric acid, the cortical granules remain intact even after fertilization, but in eggs treated with urea solution, the granules are already broken down while the eggs are still in the solution.

The present paper deals with the results of experiments designed to study the change of permeability in eggs previously treated with butyric acid or with urea solution.

MATERIALS AND METHOD

The eggs of the sea urchins, *Hemicentrotus pulcherrimus* and *Temnopleurus toreumaticus*, were used as materials. The permeability to water was measured by the photographic method described by HOBSON (1932). One drop of the egg suspension was added to 3 cc. of 40 per cent hypotonic sea water, and the eggs were photographed after three minutes. The diameter of the eggs was measured, using the photograph, and the volume of water which entered the egg during the treatment with hypotonic sea water was calculated.

Change of Permeability of the Normal Egg after Fertilization

In the first series of experiments, the change of permeability of the normal egg after fertilization was studied by Hobson's method. The experiments was carried out at room temperature (17° C.), using *Hemicentrotus* eggs. The permeability increased immediately after fertilization, and rose to the first maximum after about 3 minutes. Then it decreased to a comparatively low value. However, it again increased steadily, showing a second maximum about 15 minutes after fertilization. It remained almost unchanged until just before cleavage. Thus, the result was found to coincide well with that of Hobson.

An almost similar result was obtained with *Temnopleurus* eggs.

Experiments with Eggs Treated with Urea Solution

It has been known that urea solution is a parthenogenetic agent for the sea urchin egg. If *Hemicentrotus* eggs are immersed in a 1 M urea solution for an adequate time, the fertilization membrane is formed after their removal to sea water. But if they are immersed in the urea solution for more than 30 seconds, membrane formation does not occur after their removal to sea water, although their cortical granules are broken down. If these eggs are fertilized, they begin to develop without forming the membrane. A similar result was obtained in *Temnopleurus* eggs.

Experiments were made to compare the permeability of eggs, treated with a 1 M urea solution for 3 minutes, with that of normal eggs. It was found that the volume of water entering the eggs in 3 minutes is almost the same as that of normally fertilized eggs. Table 1 indicates one of the results of the experiments.

Table 1. Amount of water entering eggs in 3 minutes in hypotonic sea water

	Unfertilized eggs treated with urea solution for 3 minutes	Fertilized eggs after 15 minutes in control
H. pulcherrimus	370×10^3 μ^3	370×10^3 μ^3
T. toreumaticus	365×10^3 μ^3	370×10^3 μ^3

It is concluded that the permeability of unfertilized eggs treated with urea solution for 3 minutes increases to the level of the fertilized egg.

Experiments with Eggs Treated with Butyric Acid

Unfertilized eggs of *Hemicentrotus* were immersed in butyric acid-sea water (50 cc. of sea water plus 3 cc. of N/10 butyric acid) for 30 to 50 seconds at 17° C. After their return to normal sea water, the fertilization membrane was formed in almost all the eggs. But when the time of treatment with butyric acid-sea water was more than 10 minutes, the eggs lost their capacity to form the membrane.

Contrary to the experiments with urea, their cortical granules were found not to be broken down even after washing with sea water. If spermatozoa were added to the eggs, fertilization took place without membrane formation.

The change of permeability to water after fertilization was measured in eggs which had previously been treated with butyric acid-sea water for 12 minutes. It was ascertained that these eggs developed afterwards without membrane formation or breakdown of the cortical granules. The result is shown in Fig. 1, in which such eggs are compared with those of the control and eggs which had undergone the urea treatment.

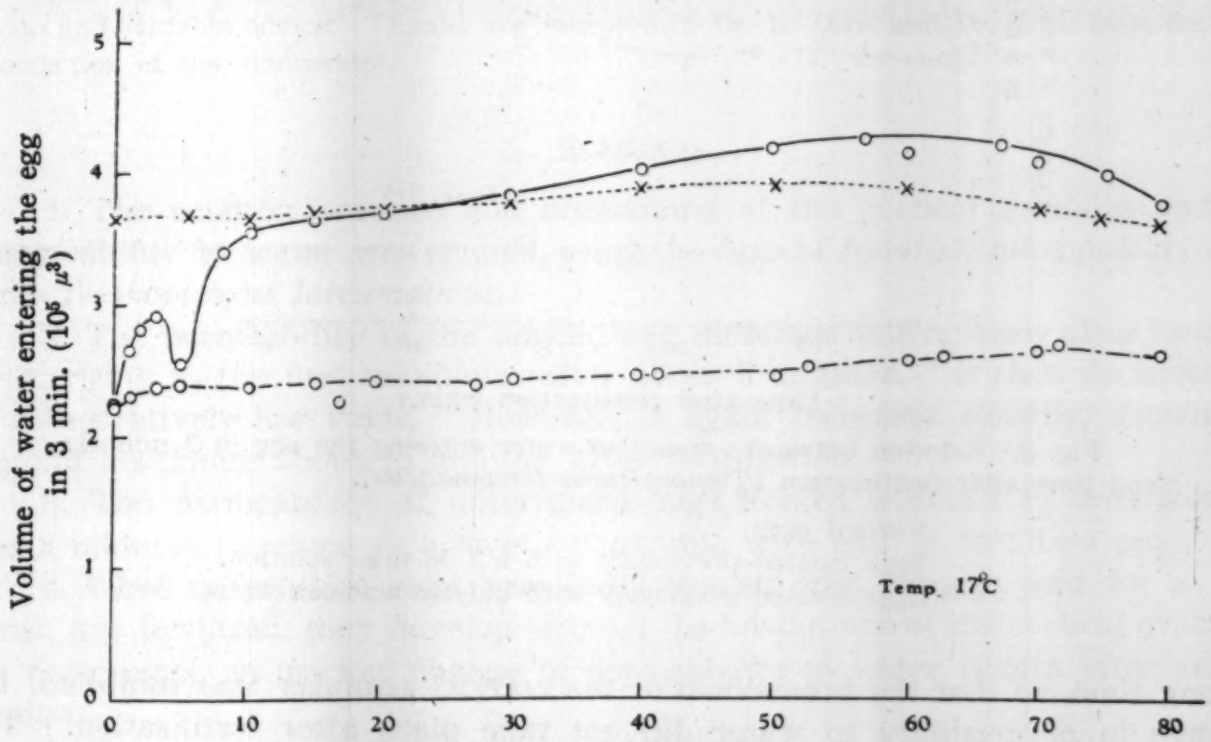

Fig. 1. Relation between amount of water entering the egg in 3 minutes and time after fertilization (*Hemicentrotus pulcherrimus*).

——— Normal egg.
..... Egg treated previously with a 1 M urea solution.
— - — Egg treated previously with butyric acid-sea water.

Similar experiments were done with *Temnopleurus* eggs. In the eggs of this species, causing membrane formation by means of butyric acid was rather difficult. They sometimes showed only 5 to 10 per cent of membrane formation even in the optimum condition. In eggs treated with butyric acid-sea water for 30 seconds and then removed to sea water, some cortical granules were found to be broken down. In eggs treated in a similar way for 40 seconds, the membrane was not formed and the cortical granules remained completely even after fertilization.

The measurement of permeability to water was therefore performed, using eggs treated with butyric acid-sea water for 40 seconds. The result is shown in Fig. 2.

It was concluded that in the eggs which were treated with butyric acid for

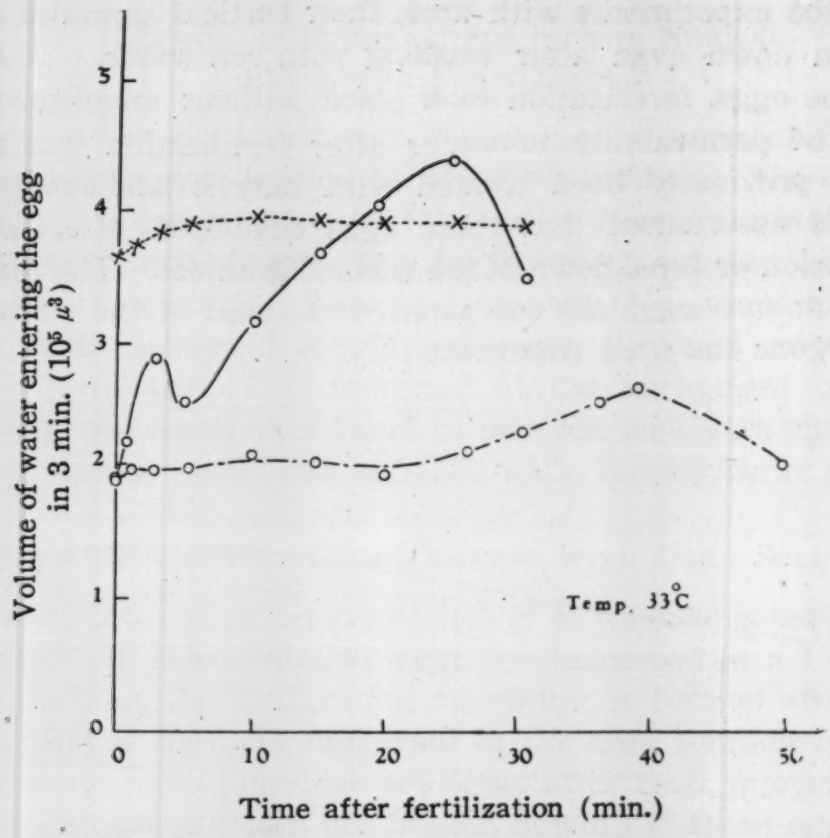

Fig. 2. Relation between amount of water entering the egg in 3 minutes and time after fertilization (*Temnopleurus toreumaticus*).

——— Normal eggs.
..... Eggs treated previously with a 1 M urea solution.
— - — Eggs treated previously with butyric acid-sea water.

a long time, so that the breakdown of the cortical granules was inhibited, the change in permeability to water did not take place after fertilization. The amount of water entering the eggs which had been thus treated was almost the same as that entering unfertilized eggs.

Discussion

As mentioned above, eggs in which the cortical granules have been broken down by the treatment with urea solution for 3 minutes show an increase of permeability comparable to that of fertilized eggs. On the contrary, eggs which have been treated with butyric acid-sea water for 12 minutes do not show any increase of permeability after fertilization. It should be noted that in such eggs, the breakdown of the cortical granules at the time of fertilization is inhibited.

On the basis of these facts, it seems probable that the increase of permeability to water is due to a reconstitution of the plasma surface following the breakdown of the cortical granules.

It has been found by Iida (1943), that a parallelism exists between the changes of electric capacitance and that of the permeability to water accompanying the fertilization of sea urchin eggs. Furthermore, Iida (1949) has reported that an increase of capacitance occurs during the breakdown of the cortical granules. These results, also, indicate the probability that an increase of per-

meability has a close relationship to the breakdown of the cortical granules.

The fact that fertilized eggs, previously treated with butyric acid for a long time, can develop without the breakdown of the cortical granules suggests the possibility that the breakdown of the cortical granules and the increase of permeability may not be essential for the initiation of development of the eggs. However, these eggs generally exhibit a marked delay in the rate of development. We are therefore unable to make any conclusive discussion concerning this problem.

The author wishes to express his sincere thanks to Dr. M. SUGIYAMA for his kind criticism and valuable advice. Thanks are also due to Dr. K. DAN and Dr. J. C. DAN for their correction of the manuscript.

SUMMARY

1. The relation between the breakdown of the cortical granules and the permeability to water was studied, using the eggs of *Hemicentrotus pulcherrimus* and *Temnopleurus toreumaticus*.

2. The permeability in the normal egg increases immediately after fertilization, rising to the first maximum after about 3 minutes. It then decreases to a comparatively low value. However, it again increases steadily, showing a second maximum about 15 minutes after fertilization.

3. The permeability of unfertilized eggs treated with a 1 M urea solution for 3 minutes increases to a level comparable with that of fertilized eggs.

4. When unfertilized eggs, previously treated with butyric acid for a long time, are fertilized, they develop without the breakdown of the cortical granules. In these eggs, no marked change of permeability to water occurs after fertilization.

5. These results indicate the possibility that an increase in permeability after fertilization has a close relation to the breakdown of the cortical granules.

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THE CHEMICAL NATURE AND THE ORIGIN OF THE CORTICAL ALVEOLI IN THE EGG OF THE MEDAKA, *ORYZIAS LATIPES*

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Introduction

Although the presence of the cortical alveoli in the unfertilized egg of fishes and their disappearance in its fertilized condition have been noticed by some investigators, little attention has been paid on their nature and significance in fertilization. Since his discovery of the cortical alveoli in the unfertilized egg of *Oryzias latipes* and their wave-like breakdown at the time of fertilization, Yamamoto (1939, 1944, 1951 *a*, 1951 *b*) has published a number of works on physiological changes in fertilization and in artificial activation. According to him the colloid content of the cortical alveoli is released in the interstice between the plasma membrane and the egg membrane at the time of the breakdown of the cortical alveoli. The egg membrane is separated by the colloid osmotic pressure of the perivitelline colloid which is derived from the cortical alveoli. Yamamoto (1951 *a*, 1951 *b*), further, found that the content of water-soluble colloid is surrounded by a lipoid wall. The lipoid nature of the wall was concluded from the fact that the wall of isolated cortical alveolus is dissolved by lipoid solvents and esterases.

The present paper deals with histochemical studies on the nature of the wall and the content of the cortical alveoli as well as the origin of them. Such studies have not been done in fish eggs. Recently Kusa (1953), in the egg of the stickleback (*Pungitius pungitius*), reported that the cortical alveolus contains a polysaccharide. He, however, has failed to observe the presence of the lipoid wall. The present study was done quite independent of him and the methods used were also partly different from those by him. Lipoid nature of the wall of the cortical alveolus was proved by Ciaccio's method and an interesting result was obtained on the origin of the cortical alveoli.

The author wished to express his thanks to Professor Toki-o Yamamoto for his kind suggestion and encouragement. He is also indebted to Mr. Eizo Nakano for his helpful advice.

Material and Method

Materials used were the oocytes and eggs of the wild type of the Medaka, *Oryzias latipes*. The belly of the egg laying females was opened and the ovary,

which contained numerous oocytes at various stages, was fixed. Fertilized eggs were obtained as usual (*cf.* YAMAMOTO 1944 etc.).

Polysaccharide: The histochemical method used to detect the polysaccharide nature of the content in the cortical alveoli was that developed by HOTCHKISS (1948) and LILLIE (1948), and a solution of 2, 4 dinitrophenylhydrazine was used to stain the polysaccharide besides SCHIFF's reagent. Materials were fixed with BENSLEY's solution,[1] and paraffine sections were made as usual. They were run through alcohols to distilled water and immersed in sodium metaperiodate solution[2] for 15–20 minutes. After this pre-treatment and successive washing in water, these sections were immersed in 50 per cent alcohol solution of 2, 4 dinitrophenylhydrazine for 1–1.5 hours, and washed in water, then placed in SCHIFF's reagent for 5–10 minutes. Then they were washed in SO_3 water, dehydrated and mounted in balsam.

Lipoid: CIACCIO's method (cited from MORI 1949) was employed to demonstrate the lipoid wall of the cortical alveoli.

Materials were fixed with the following solution for 2 days:

5% Potassium bichromate	80
40% Formalin	20
Glacial acetic acid	5

They were, then, immersed in 3 per cent potassium bichromate solution for about 10 days at 37° C. in the dark. This solution was renewed every other day. Following these procedures, usual paraffine sections were prepared. After being deparaffinized in xylol, sections were brought through absolute alcohol to the following solution for staining:

80% Alcohol	95	saturated with Suden III
Acetone	5	

The sections were then mounted in glycerine.

RESULT

By the double-staining method described above, three of the polysaccharide containing structures; germinal vesicle, cortical alveoli and yolk were stained yellow, reddish-violet and violet, respectively. Non-pretreated materials were not stained with both reagents. In the early oocyte, only the germinal vesicle is stained, and as the growth proceeds, many vacuole-like bodies begin to appear around the germinal vesicle, and spread over the ground cytoplasm, coming to the nodulous appearance. This seems to be the original phase of the cortex of the mature egg and may be termed as "pre-cortex." Thereafter the yolk begins to increase, and at the same time, reddish-violet droplets appear in the nodulous

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[1] 5 g. sublimate and 2.5 g. potassium bichromate in 100 cc. distilled water with 10 cc. formalin added before use.

[2] 1 g. crystal sodium metaperiodate in 100 cc. distilled water added 0.5 cc. 70% HNO_3 (s.g. 1.42).

"pre-cortex," scattering at random (Fig. 1). The more accumulation of yolk is advanced, the thinner becomes the pre-cortex, probably be due to the internal pressure of the growing yolk. Throughout this process, reddish-violet droplets are moved peripherally accompanying with the pre-cortex, and at last they become to monolayer in the cortex (Fig. 2, 3, 4). The cortex of the matured egg is thus formed. In the fertilized egg, on the other hand, reddish-violet droplets disappear completly and the cortex shows a simple constitution without any optical structures (Fig. 5), indicating that the reddish-violet droplets are nothing but the cortical alveoli of the mature unfertilized egg.

CIACCIO's method employed enabled us to observe the orange coloured wall around the cortical alveoli. They can be seen black in the photographs (Fig. 6, 7). It is certain that they are nothing but the lipoid wall, the existence of which was ascertained by YAMAMOTO already by solubility test. In the fertilized egg, only the oil drops, which were slightly condensed together after fertilization, are distributed in the cortex of the egg (Fig. 8).

DISCUSSION

The polysaccharide nature of the content of the cortical alveoli may be a common fact in many kinds of animals. KUSA (1952) found a HOTCHKISS positive nature in the cortical alveoli of the egg of the stickleback, *Pungitius pungitius*. In sea urchin eggs too, such a nature of the cortical granules was found by MONNÉ and SLAUTTERBACK (1950), who argued that these granules contain a mucopolysaccharide with sulfate residue. The conclusion is based upon the fact that these granules have same staining property as that of jelly coat which is consist of mucopolysaccharide with sulfate residue.

Though clear-cut demonstration of the lipoid wall of cortical alveoli or cortical granules in other kind of eggs has not yet been made, observations in the cortical granules of sea urchin eggs by MCCULLOCH (1952, electron microscopic) and by ENDO (1952, phase contrast microscopic), which showed two phases in the cortical granule, are interesting in this connection.

YAMAMOTO (1951 *a*) clearly showed that the cortical alveolus of *Oryzias* egg is composed of the content which contains water-soluble colloid and its lipoid wall. The latter was based on the solubility test of the isolated cortical alveoli by lipoid solvents and esterases. He also found the reddish granules around the cortical alveoli, and observed that these granules, which were named as cortical granule A, became indistinct in contour at the time of fertilization and activation, as though they were dissolved or broken down. Hence, he suggested that these granules may be bearers of esterase system and that they may be activated in consequence of fertilization and artificial activation. His conclusion that the wall of the cortical alveoli is lipoid nature has been proved by CIACCIO's method in the present study. It is concluded that the cortical alveoli in the unfertilized egg of *Oryzias latipes* is composed of lipoid wall and the content which is a polysaccharide in nature.

The origin of the cortical alveoli in the egg of the fish is something different from that of the cortical granules of sea urchin egg (MOTOMURA 1936, RUNNSTRÖM and MONNÉ 1948, MONNÉ and HÅRDE 1951, MCCULLOCH 1952). The

latter are formed in the protoplasm and migrate towards the cortex as the maturation proceeds,[3] and the former, as described above, are originated in the "pre-cortex" and move peripherally accompanying with the "pre-cortex," and are embedded in the cortex as the maturation is completed.

Summary

The chemical nature and the origin of the cortical alveoli in the ova of the Medaka, *Oryzias latipes*, were studied by the histochemical methods, and the following results were obtained:

1) The content of the cortical alveoli are of polysaccharide nature, which is demonstrated by periodate-SCHIFF's method.

2) They have a lipoid wall which is evidenced by CIACCIO's method.

3) They are originated in a nodulous region in the early oocyte, which is named as "pre-cortex," and migrated peripherally as the maturation proceeds.

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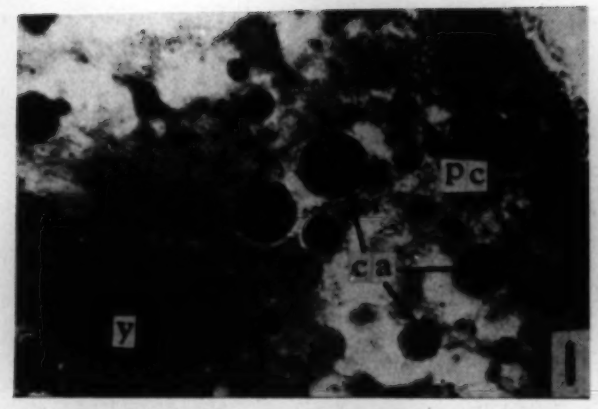
[3] Same fact was reported by MOTOMURA (1952) on the cortical granules in the egg of *Rana nigromaculata*.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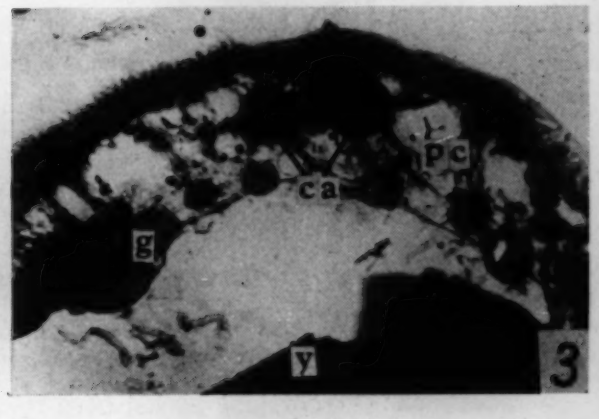
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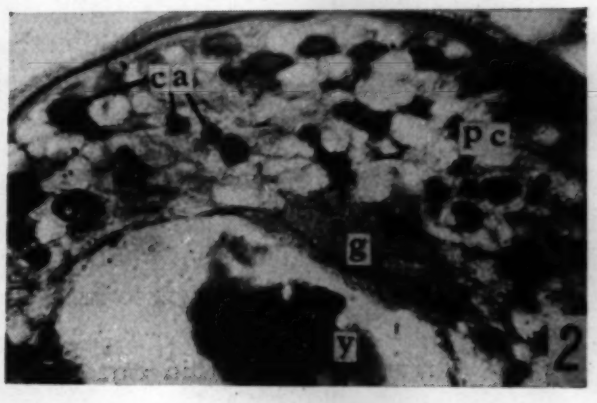
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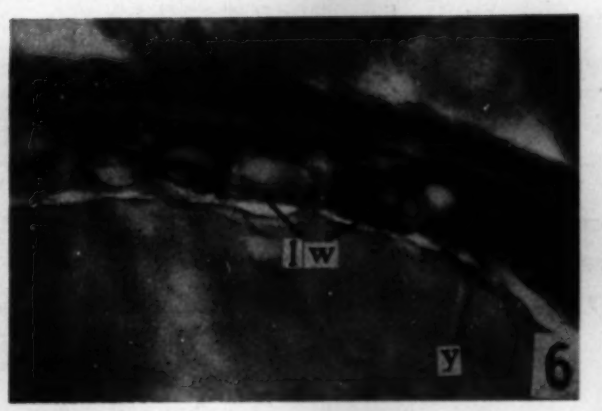
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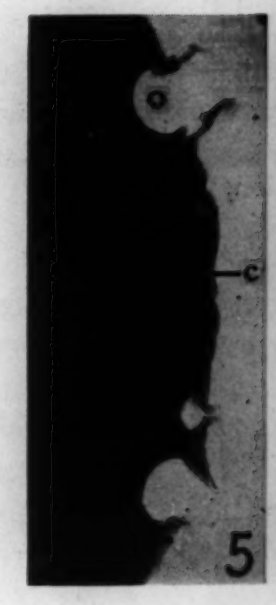
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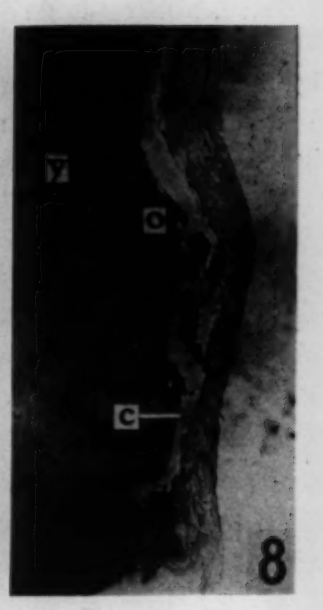
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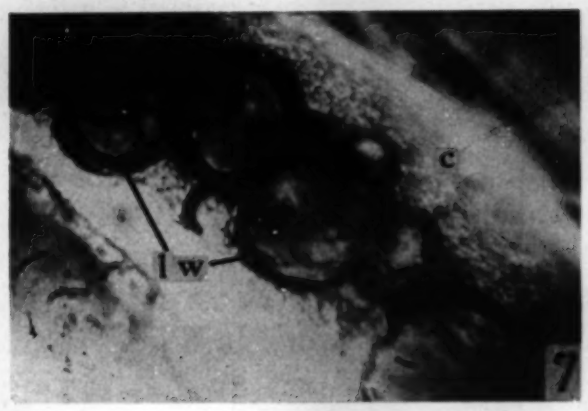
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Explanation of Figures
(Plate 3)

c:····cortex, ca:····cortical alveolus, g:····germinal vesicle, o:····oil drop, pc:····pre-cortex, lw:····lipoid wall of the cortical alveolus, y:····yolk.

Fig. 1-5. Staining of *Oryzias* oocytes and eggs by 2, 4 dinitrophenylhydrazine-SCHIFF method. Three structures with polysaccharide, germinal vesicle, cortical alveoli and yolk, are stained yellow, reddish-violet and violet, respectively.

Fig. 1. Oocyte. The cortical alveoli appeared in the nodulous "pre-cortex" at random. ×150

Fig. 2, 3. Oocyte. More accumulation of the yolk is advancing, and parallely the cortical alveoli is becoming to thinner arrangement. The germinal vesicle attaches to inner side of the pre-cortex. The yolk is shrinked by the treatment. ×150

Fig. 4. Mature unfertilized egg. The cortical alveoli are arranged in the thin cortex, making a monolayer. ×150

Fig. 5. Just fertilized egg. The cortical alveoli disappeared. Oil drops condensed together at fertilization were dissolved by the treatment. The egg membrane was denuded to make the treatment easier. ×150

Fig. 6-8. Staining of *Oryzias* oocytes and eggs by CIACCIO's method. The lipoid wall of the cortical alveoli and the oil drops are stained orange.

Fig. 6, 7. Mature unfertilized egg. The cortical alveoli with the lipoid wall are embeded in the cortex, forming a monolyer. Fig. 6. ×150 Fig. 7. ×300

Fig. 8. Just fertilized egg. Nothing can been seen in the cortex except oil drops condensed together at fertilization. ×150.

RESPIRATORY METABOLISM DURING FISH DEVELOPMENT

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Although numerous investigations have been done on the respiration of fish eggs during early development, relatively few attempts have been made to analyze the respiratory metabolism. AMBERSON and ARMSTRONG (1933) have shown in *Fundulus* eggs that the respiratory quotient is high in the first day and it declines continuously to lower values during development. This indicates that the egg uses first carbohydrates, then proteins and fats in the order presented by NEEDHAM (1931). On the other hand, BRACHET (1934) reported in the frog egg that the respiratory quotient has a low value during segmentation and this value increases sharply at the beginning of gastrulation. Similar results have been obtained by ÖHMAN (1940) and HUTCHENS, KELTCH, KRAHL and CLOWES (1942) in the sea urchin egg. Furthermore, TRIFONOVA and her collaborators (1937, 1939) have shown in the eggs of the perch that cyclic changes of respiration and glycolysis take place between fertilization and neurulation. In *Fundulus* eggs, however, such fluctuations are not found at least in respiration where increases have been shown to occur continuously (PHILIPS 1940). Thus, the results of these investigations remain somewhat contradictory regarding the respiratory changes during fish development. The purpose of the present study is to try and resolve this problem, using eggs of the medaka, *Oryzias latipes*.

Before going further, the writers wish to express their gratitude to Prof. T. YAMAMOTO for his encouragement and valuable advice. Thanks are also due to Dr. J. ISHIDA of Tokyo University for his helpful suggestion and criticism.

MATERIALS AND METHODS

Materials. Materials used were eggs of the orange-red variety of the medaka, *Oryzias latipes*. Adult females lay eggs daily for a considerable period during spring and summer. Spawned eggs hang in a tiny cluster from the female's vent and they may be removed by suction of a wide-mouthed pipette. For the experiments, eggs were washed with distilled water and were separated one by one. This procedure is necessary to improve the synchrony of development. Then, eggs were allowed to develop in the isotonic RINGER's solution (M/7.5 NaCl 100 parts, M/7.5 KCl 2.0 parts, M/11 $CaCl_2$ 2.1 parts, M/10 $NaHCO_3$ 0.25 parts, pH 7.3) until desired stages were reached at room temperature (25-28° C.). At 25° C., the embryos hatch out in 10 days. The cleaving eggs develop to the late gastrula stage at the end

of the first day. The optic vesicle stage is reached during the second day. In the third day the circulation of the blood begins. For the details of the daily development, ISHIDA's paper (1944) or RUGH's book (1948) should be consulted.

Enzymatic activities. The oxygen uptake was measured in WARBURG manometers, with a vessel capacity of about 5 ml. 0.2 ml. of 10 per cent KOH was placed in the center cup. For the determination of the cyanide inhibition, the KOH was replaced by KREBS' (1935) KCN-KOH mixture. The temperature was regulated to 30° C. and the shaking rate was 100 to 110 cycles per minute with a 4 cm. amplitude. The gas space was filled with air.

In the experiments with intact eggs, 100 to 300 eggs were suspended in M/50 RINGER's solution so that the total volume was 2 ml. and were transferred to each vessel. The pH of media was adjusted to 7.3 by the addition of phosphate buffer. For the preparation of homogenates, 2,500 eggs were washed with isotonic KCl and were then kept for 5 minutes in an ice bath. The material was then ground in an ice-chilled homogenizer and was diluted with M/7.5 KCl solution (pH 7.3). The extract was prepared by slow centrifugation of homogenates and the opalescent supernatant fluid was used as the enzyme preparation. 1.5 ml. of this preparation, which is equivalent to 500 eggs, was used in each vessel.

Oxidative phosphorylation was estimated according to the method of CROSS, TAGGART, COVE and GREEN (1949). The reaction mixture adopted for use in the present experiment has the following composition;

> 0.3 ml. 0.1 M Na-succinate, 0.5 ml. 0.1 M phosphate buffer, 0.3 ml. 0.01 M Na-ATP, 0.2 ml. 0.1 M $MgCl_2$, 0.1 ml. 1.0 M fructose, 0.1 ml. 0.33 M NaF and 1.0 ml. homogenate.

The pH of the mixture was 7.3. Hexokinase was not added. The phosphate content was determined by the method of FISKE and SUBBAROW (1925).

Cytochrome oxidase activity was estimated according to the method of SCHNEIDER and POTTER (1943) by measuring oxygen uptake in a system in which reduced cytochrome C was supplied in excess by the addition of ascorbic acid. $CaCl_2$ was added as activator and the pH of the mixture was maintained to 7.3 by the addition of phosphate buffer. Succinoxidase activity was determined according to the same method (SCHNEIDER and POTTER 1943), except that Na-ascorbate was substituted for Na-succinate and $AlCl_3$ was further used as activator. The optimum pH was found to be between 7.0 and 8.0 in both cases, which is in quite accordance with the data from other materials.

The respiratory quotient of intact eggs was mostly determined by the direct method of DIXON (1943). In some experiments, the indirect method of WARBURG and YABUSOE (1924) was used.

Anaerobic glycolysis was determined according to the method described in UMBREIT, BURRIS and STAUFFER (1949). The reaction mixture was slightly modified and had the following composition;

> 0.3 ml. 0.1 M glucose, 0.1 ml. 0.15 M K-pyruvate, 0.1 ml. 0.05 M phosphate buffer (pH 7.3), 0.15 ml. 0.5 M $KHCO_3$, 0.1 ml. 0.01 M Na-ATP, 0.2 ml. 0.1 per cent DPN, 0.15 ml. 0.05 per cent K-HDP, 0.15 ml. 0.1 M $MgCl_2$, 0.2 ml. 0.1 M KF and 0.1 ml. homogenate.

Chemical analyses. Glycogen was separated by the alkaline hydrolysis and precipitation method of LINDBERG (1945), and then determined by the method of BOETTIGER (1946). In some experiments, the reducing sugar was determined by the method of FOLIN and MALMROS (1929) after acid hydrolysis. The results obtained by both methods agree almost well in absolute values for glycogen. Lactic acid was estimated according to the method of BARKER and SUMMERSON (1941).

Diphosphopyridine nucleotide was determined according to the method of JANDORF, KLEMPESER and HASTING (1941). As the enzyme source, however, water extract of the acetone powder of cat muscle was used. 300 eggs were homogenized and extracted with 60 per cent alcohol. The extracts were evaporated to dryness and extracted three times with water. Aliquots of the water extracts were assayed in terms of its ability to serve as the active group of phosphoglyceraldehyde and glycerophosphate dehydrogenases.

Thiamin was estimated according to the fluorometric method of HENNESY and CERECEDO (1939). 600 eggs were dropped into 10 ml. of boiling M/20 acetate buffer (pH 4.5) and heated for a few minutes in order to destroy the enzyme action. They were then homogenized, diluted with distilled water and divided into two portions. One was assayed immediately for free thiamin and the other digested with Taka-diastase and then assayed for total thiamin.

The determinations of flavin were made essentially according to YAGI (1953). The digestion method by Taka-diastase was principally the same as that described in thiamin assay.

Sources of special chemicals. Diphosphopyridine nucleotide (DPN) was prepared by the method of LE PAGE (1947). Adenosine triphosphate (ATP), obtained from dog muscle according to the method of SZENT-GYÖRGYI, was purified by the method described in Biochemical preparations (1949). Hexose diphosphate (HDP) was prepared by the method of NEUBERG and LUSTIG (1942). Cytochrome C was isolated from beef heart muscle by the method of KEILIN and HARTREE (1937). Na-pyruvate was prepared from freshly distilled pyruvic acid by the method of ROBERTSON (1942). Other chemicals were obtained from commercial sources.

RESULTS AND DISCUSSION

Oxygen uptake. It has been shown in previous paper (NAKANO 1953) that the oxygen uptake of *Oryzias* eggs increases gradually as an exponential function with time during the initial post-fertilization period. The following experiments were performed with the eggs throughout development up to hathing.

The results are graphically shown in Fig. 1. The rate of oxygen uptake gradually increases as development proceeds. In agreement with PHILIPS (1940), there is no evidence of any fluctuation in the oxygen uptake during gastrulation and neurulation, unlike the work of TRIFONOVA (1937). However, the rise in the rate of oxygen uptake between second and third day is more rapid than that before second or after third day. It seems that this increase may be associated with the beginning of the circulation. These results are in quite

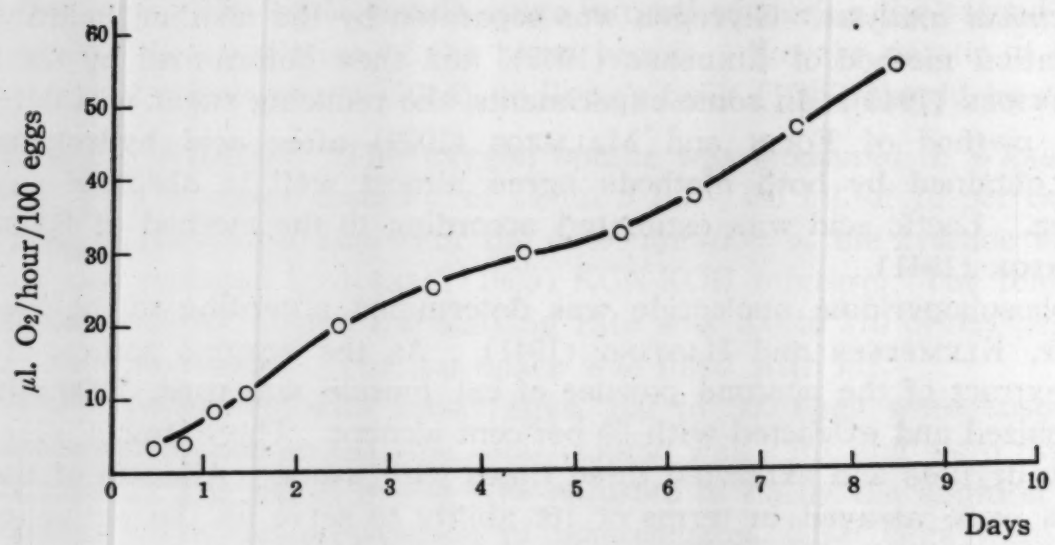

Fig. 1. Oxygen uptake during development.

accordance with a number of studies made by other authors (notably AMBERSON and ARMSTRONG 1933 in *Fundulus* and ISHIDA 1952 in *Oryzias*).

The oxygen uptake of *Oryzias* eggs is strongly inhibited by cyanide and azide. On the contrary, dimethyl-*p*-phenylendiamine can increase the oxygen uptake of intact eggs (Table 1). These results suggest that the respiration of

Table 1. Effect of cyanide, azide and dimethyl-*p*-phenylendiamine on the oxygen uptake of intact eggs.
Materials: first day eggs, pH 7.3 or 6.2, 30° C.

Additions	Concentration	Oxygen uptake μl./hour/1,000 eggs
Cyanide	0	29.3
	M/5,000	20.2
	M/2,000	10.3
	M/1,000	4.4
Azide	0	33.0
	M/1,000	27.4
	M/500	18.5
	M/100	11.8
Dimethyl-*p*-phenylen-diamine	0	27.8
	M/1,000	45.2

Oryzias eggs may be catalyzed in the main by ordinary cytochrome system. Similar conclusion has been deduced by PHILIPS (1940) concerning *Fundulus* eggs. It was also found that intermediates of tricarboxylic acid cycle do not influence the oxygen uptake of intact eggs. This may be due to low permeability of the plasma membrane to each of these substances. Hence the experiments were performed with the egg homogenates.

The endogenous oxygen uptake of homogenates was very low and its rate tends to decline with time. Therefore, the rates of oxygen uptake were determined in the earlier constant rate portion. When the intermediates of tricarboxylic acid cycle, such as citrate, succinate, malate, glutamate and pyruvate,

were added to homogenates, there was a marked increase in the oxygen uptake (Table 2). Among them, succinate exceedingly stimulated the oxygen uptake.

Table 2. Effect of tricarboxylic acid cycle intermediates on the oxygen uptake of egg homogenates. Concentration of substrate: M/150, pH 7.3, 30° C.

Substrate	Oxygen uptake μl./hour	
	first day eggs	third day eggs
None	1.8	3.8
Citrate	5.9	10.5
Succinate	16.1	20.4
Malate	5.8	10.2
Glutamate	5.8	11.3
Pyruvate	4.5	11.0

This seems to depend upon the fact that the succinoxidase is the hardiest of the oxidative enzymes in the cyclophorase system. It may be remarked that the addition of adenosine triphosphate to homogenates stimulates the oxidation of substrates. In the oxidation of succinate, however, adenosine triphosphate did not influence on the oxygen uptake. These results are in good accordance with data from rabbit kidney (CROSS, TAGGART, COVO and GREEN 1949).

It is also worthy of note that the endogenous oxygen uptake of homogenate increases between first and third day. The same is true when the substrate is added to homogenate. It may be assumed that the enzymes in tricarboxylic acid cycle are synthesized during early development. This is, however, at variance with the results of SPIEGELMAN and STEINBACH (1945), who reported that the oxygen uptake exhibited by the brei of frog eggs decreases as development proceeds. In addition, the brei of frog eggs has a considerably higher metabolism than intact egg during early embryonic stages. SPIEGELMAN and STEINBACH concluded that the respiratory enzyme of frog eggs is not saturated by its substrate at the beginning of development and that increase in oxidation results from saturation of the respiratory enzyme without any synthesis of the latter. On the basis of the present experiments, this is not true for *Oryzias* eggs. Further evidence in this connection will be found below.

In any case, it is quite possible that tricarboxylic acid cycle is present in *Oryzias* eggs. The evidence for the occurrence of this cycle in the egg has been discussed by KELTCH, STRITTMATTER, WALTERS and CLOWES (1950) and CLELAND and ROTHSCHILD (1952) in the sea urchin.

Oxidative phosphorylation. Although no attempt, hitherto, has been made to ascertain the occurenence of oxidative phosphorylation in vertebrate eggs, it seems valid to assume that this mechanism plays an important role in development of *Oryzias* eggs. Preliminary experiments indicate that the egg homogenates are capable of effecting oxidative phosphorylation (Table 3). Furthermore, it was found that 2,4-dinitrophenol completely inhibits oxidative phosphorylation without depressing the rate of succinate oxidation. Similar results have been obtained by KELTCH, STRITTMATTER, WALTERS and CLOWES (1950) in egg homogenates of the sea urchin.

Table 3. Effect of 2,4-dinitrophenol on the oxidative phosphorylation. Homogenates: first day eggs, substrate: M/150 succinate, pH 7.3, 30° C.

Additions	Oxygen uptake μl./hour	phosphate uptake μg./hour	P : O ratio
None	16.2	39	0.87
10^{-4} M DNP.....	15.3	10	0.23

Cytochrome oxidase and Succinoxidase. The experiments described above indicate that both cytochrome system and tricarboxylic acid cycle are present in *Oryzias* eggs. The question now arises as to changes of these systems during development. Measurements were performed on the activities of cytochrome oxidase and succinoxidase throughout development. The results are illustrated in Figs. 2 and 3. Activities of these two enzymes increase gradually during the course of development, following an exponential curve that is parallel to the increase in the oxygen uptake. It is clear that these two enzymes are synthesized even in earlier stages of development. This does not agree with the results of SPIEGELMAN and STEINBACH (1945). They reported

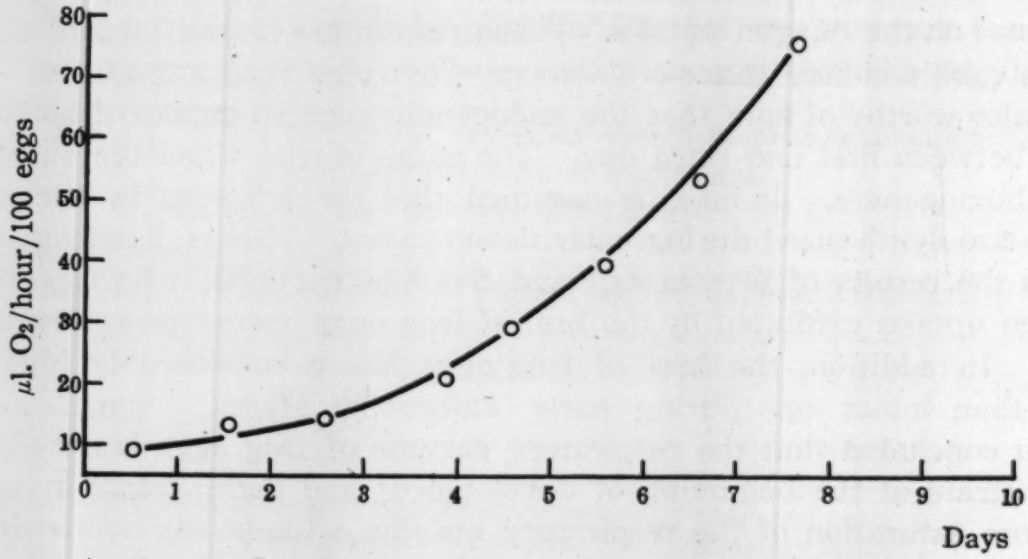

Fig. 2. Cytochrome oxidase activity during development.

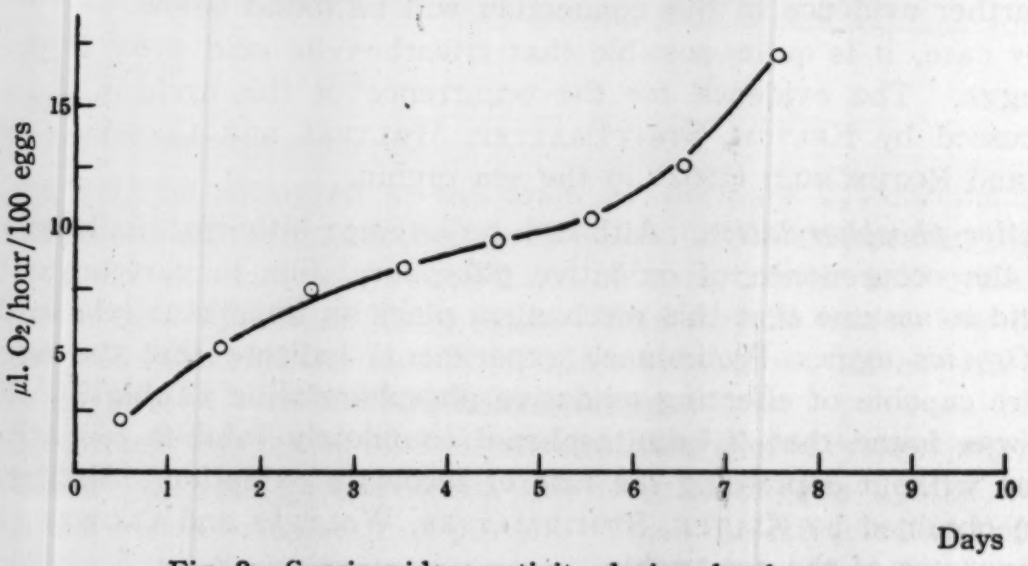

Fig. 3. Succinoxidase activity during development.

that cytochrome oxidase remains uniformly high during development up to the stage of heart beat. These apparent discrepancies may be due to species difference. For later stages of development, however, the results of BOELL (1950) with *Amblystoma* agree with those of the present study in showing a continuous rise in the activities of cytochrome oxidase and succinoxidase. According to BOELL, the synthesis of these enzymes represents the synthesis of metabolically active cytoplasm at the expense of the yolk. In *Oryzias* eggs, we cannot say with certainty that the rise in enzyme activity is really correlated with the utilization of the yolk.

It is worthy of note that the oxygen uptake by cytochrome oxidase amounts to about two times that of intact eggs at the beginning of development. This indicates that the fertilized egg of *Oryzias* contains an amount of cytochrome oxidase sufficient to catalyze such higher rates of oxidation than normal. Normally the enzyme is probably far from being saturated by its substrates. SPIEGELMAN and STEINBACH (1945) and BOELL (1950) claimed the similar conclusion from studies in amphibian eggs.

Respiratory quotient. The respiratory quotient of intact eggs was measured from the first to the 9th day of development. As shown in Fig. 4, the respiratory quotient has a low value at the beginning of the first day. But this value increases sharply during gastrulation and shows maximum in the second day. These results suggest that an oxidation of substances other than carbohydrate may occur in the *Oryzias* eggs up to the gastrula stage and that at this time the metabolism of carbohydrate may begin. Similar results have been obtained by BRACHET (1934) in the frog as well as by ÖHMAN (1940) and HUTCHENS, KELTCH, KRAHL and CLOWES (1942) in the sea urchin. BRACHET considered that the nature of oxidations is modified during gastrulation. On the other hand, AMBERSON and ARMSTRONG (1933) have shown in *Fundulus* eggs that the respiratory quotient is high in the first day and declines gradually to lower values during later development. They postulated that a sequence in utilization of carbohydrates, proteins and fats is present during embryonic development. The discrepancy between their result and our one may be sought an

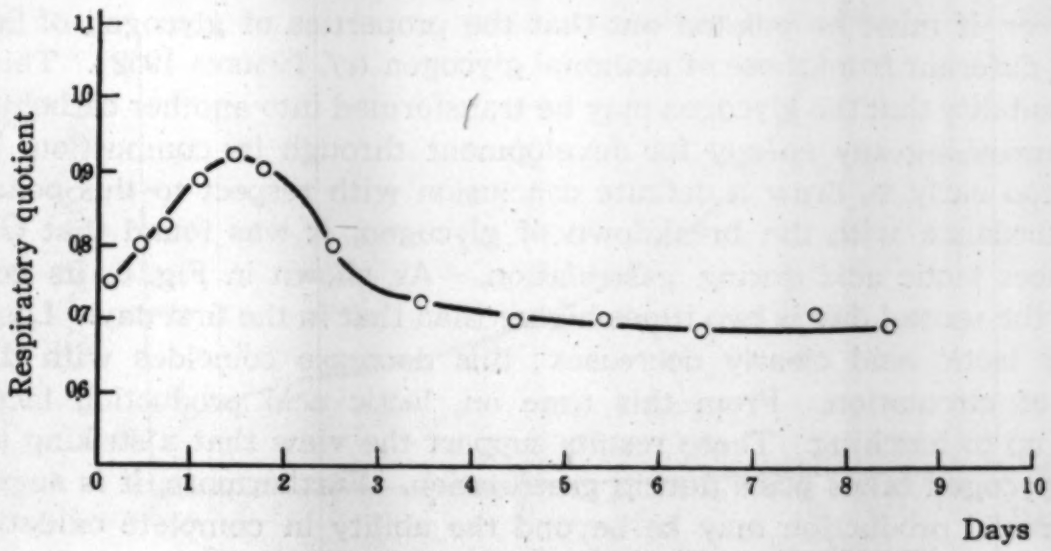

Fig. 4. Respiratory quotient during development.

explanation of the fact that they were unable to measure during segmentation, when the respiratory quotient of *Oryzias* eggs has an exceptionally low value. It may be added that the carbohydrate phase in *Oryzias* egg is confined to the second day and is very short in comparison with that shown in the frog egg. In this respect, the present results are in agreement with those of AMBERSON and ARMSTRONG.

Glycogen and lactic acid. Although the interpretation of respiratory quotient is often erroneous, it may be postulated that at the end of gastrulation the catabolism is principally carbohydrate. In this connection it is important to know whether or not the amount of glycogen decreases during gastrulation. Analyses were carried out on the glycogen content of *Oryzias* eggs during the course of development. The results are shown in Fig. 5. It is seen that the glycogen content is apparently reduced in the second day and then it decreases gradually. Similar results have been obtained by BRACHET and NEEDHAM (1935) in *Rana* and by OOI (unpublished data) in *Oryzias*. This is in agreement with the interpretation given for the respiratory quotient.

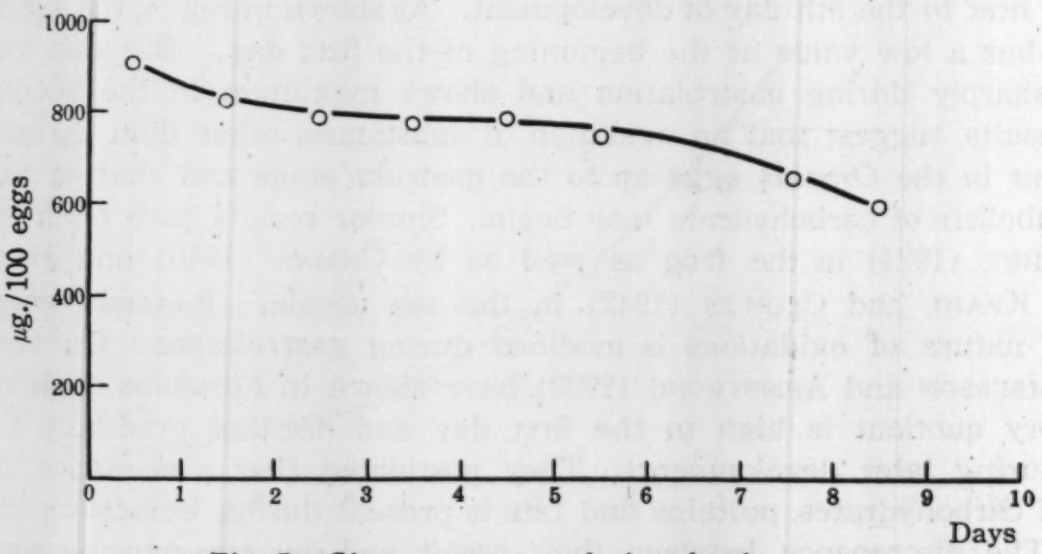

Fig. 5. Glycogen content during development.

However, it must be pointed out that the properties of glycogen of fish are somewhat different from those of mammal glycogen (*cf.* IMMERS 1952). This suggests a possibility that the glycogen may be transformed into another carbohydrate without furnishing any energy for development through its combustion, but it is as yet too early to draw a definite conclusion with respect to this point.

In accordance with the breakdown of glycogen, it was found that *Oryzias* egg produces lactic acid during gastrulation. As shown in Fig. 6, its concentration in the second day is two times higher than that in the first day. Later the amount of lactic acid clearly decreases; this decrease coincides with the appearance of circulation. From this time on, lactic acid production increases gradually up to hatching. These results support the view that a striking breakdown of glycogen takes place during gastrulation. Furthermore, it is suggested that lactic acid production may be beyond the ability in complete oxidation of pyruvic acid in earlier stages of development and that disappearance of accumu-

lated lactic acid after the beginning of the circulation may be due to the elaboration of tricarboxylic acid cycle for the complete oxidation of pyruvic acid. The fact that the rate of oxygen uptake increases sharply in the third day seems to support these assumptions.

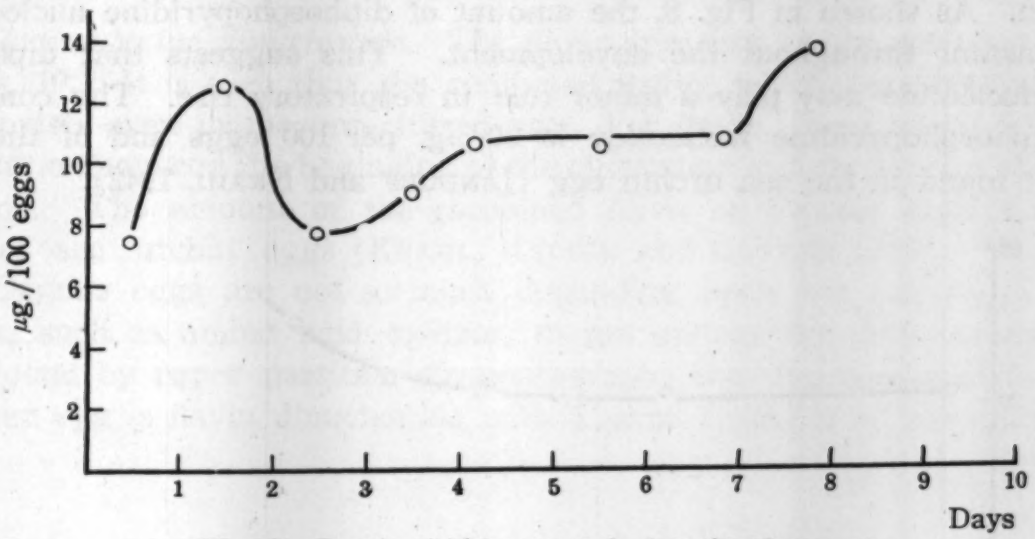

Fig. 6. Lactic acid content during development.

According to TRIFONOVA, KOROVINA and SLIUSSAREV (1939), the cyclic changes of lactic acid content take place between fertilization and the neurulation of eggs of the perch. However, it seems unlikely that similar chages occur in *Oryzias* eggs.

Anaerobic glycolysis. In relation to the breakdown of glycogen it is of interest to investigate the activity of anaerobic glycolysis during development. The measurements were made on eggs from first to 8th day. The results are shown in Fig. 7. It is seen that glycolytic activity increases uniformly throughout development. There is no abrupt rise in the activity as might be expected from the results of the lactic acid production. It is, however, worthy of note that the determinations made on the anaerobic glycolysis do not agree perfectly with those on the aerobic production of lactic acid.

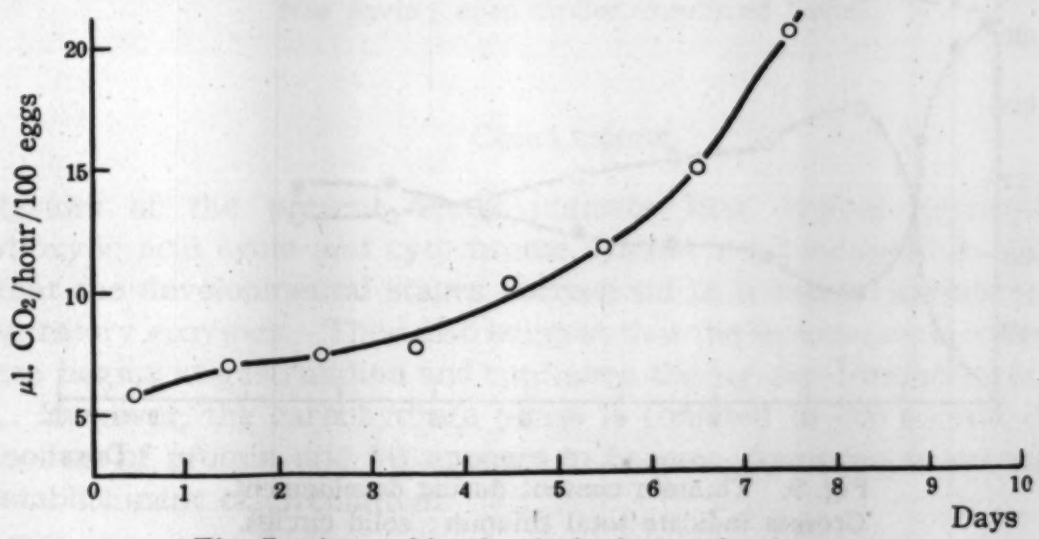

Fig. 7. Anaerobic glycolysis during development.

Preliminary experiments show that iodoacetamide and *p*-chloromercuric benzoate completely inhibit this activity.

Diphosphopyridine nucleotide. It is worth while to compare the amount of diphosphopyridine nucleotide in developing eggs with activities of glycolysis and respiration. As shown in Fig. 8, the amount of diphosphopyridine nucleotide is rather constant throughout the development. This suggests that diphosphopyridine nucleotide may play a minor role in respiratory rise. The concentration of diphosphopyridine nucleotide is 20 μg. per 100 eggs and of the same order that found in the sea urchin egg (JANDORF and KRAHL 1942).

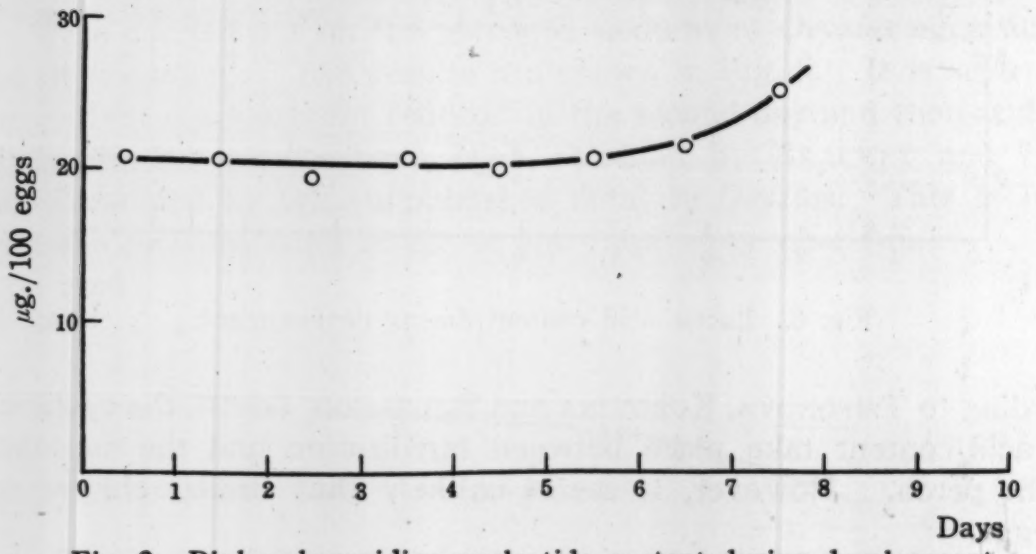

Fig. 8. Diphosphopyridine nucleotide content during development.

Thiamin. Free and total thiamin were separated by digestion method and then estimated. The results are shown in Fig. 9. Total thiamin of *Oryzias* eggs is about 0.2 μg. per 100 eggs and remains unaltered during the earlier development. The amount of phosphothiamin, however, increases as gastrulation proceeds, while that of free thiamin decreases. It is noteworthy that there is

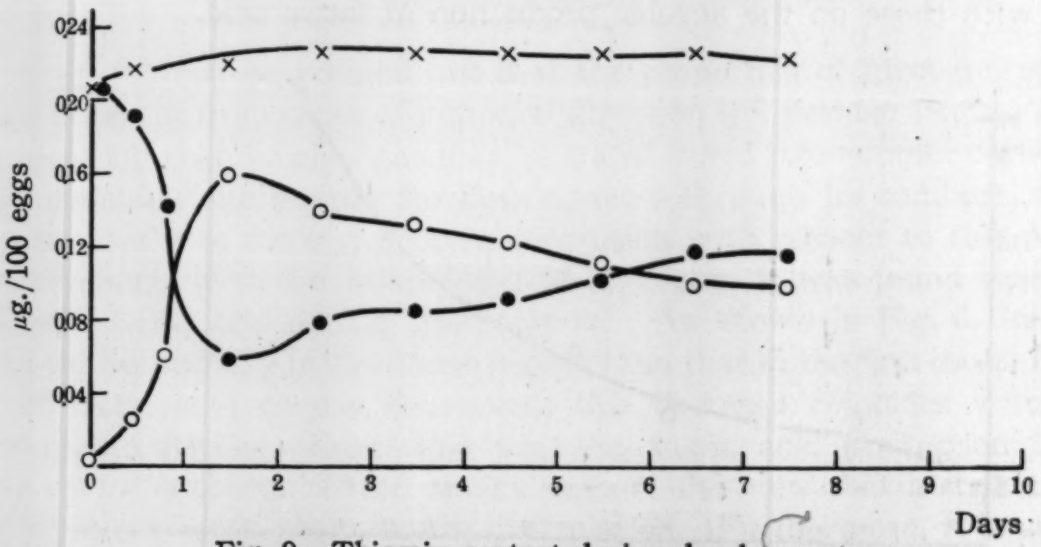

Fig. 9. Thiamin content during development. Crosses indicate total thiamin; solid circles, free thiamin; open circles, phosphothiamin.

no phosphothiamin in the unfertilized egg. These results indicate that *Oryzias* eggs synthesize phosphothiamin during gastrulation. In view of the role of phosphothiamin in carbohydrate metabolism, it is interesting that there is a comparable increase in glycogenolysis and synthesis of phosphothiamin during gastrulation.

Flavinadenine dinucleotide. The flavin contents of *Oryzias* eggs are shown in Fig. 10. It is seen that the combined flavin, which may be flavin nucleotide, exists even in the unfertilized egg. For flavin, there seems to be a close correlation between the beginning of the circulation and the synthesis of flavin nucleotide. The amount of the combined flavin of *Oryzias* eggs is lower than that of sea urchin eggs (KRAHL, KELTCH and CLOWES 1940). This suggests that *Oryzias* eggs are not so much depending upon the activity of flavin enzymes, such as amino acid oxidase, to get energy for development. It was also found by paper partition chromatography that the combined flavin of the ferilized egg is flavin dinucleotide with a small quantity of flavin mononucleotide.

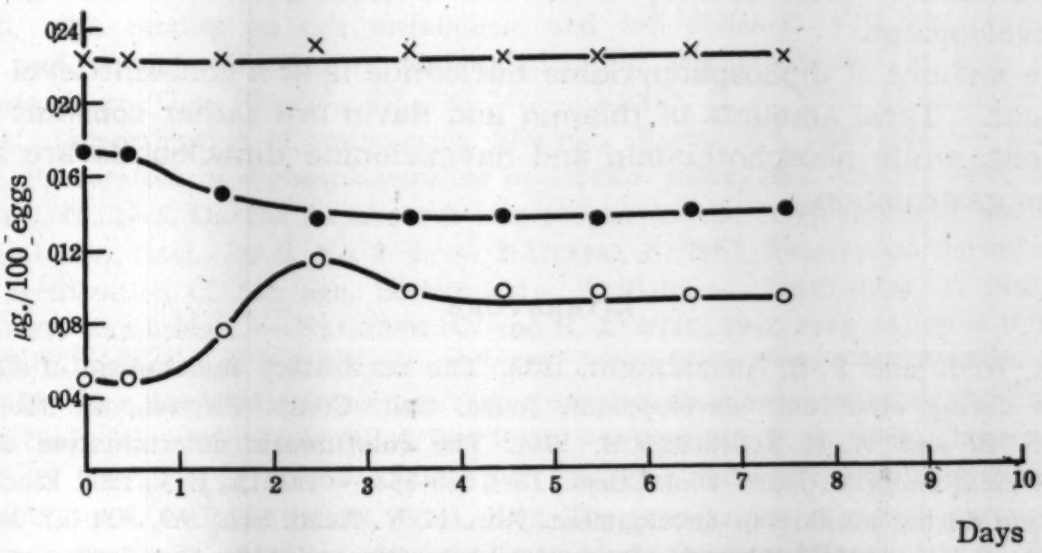

Fig. 10. Flavin content during development. Crosses indicate total flavin; solid circles, free flavin; open circles, combined flavin.

CONCLUSIONS

Results of the present study indicate that typical glycolytic system, tricarboxylic acid cycle and cytochrome system are functional in *Oryzias* eggs, and that the developmental stages correspond to a rather harmonious increase in respiratory enzymes. They also suggest that the intense metabolism of carbohydrate begins at gastrulation and continues during the formation of embryonic body. However, the carbohydrate phase is confined to the second day and the metabolism of protein and fat appears to become dominant in coincidence with the establishment of circulation.

Summary

1. The oxygen uptake of *Oryzias* eggs increases exponentially during the course of development. The oxygen uptake of intact eggs is inhibited by cyanide and azide, while increased by dimethyl-*p*-phenylendiamine. The addition of intermediates of tricarboxylic acid cycle, such as citrate, succinate, malate, glutamate and pyruvate, stimulates the oxygen uptake of egg homogenates.

2. The phosphorylation is increased by the addition of succinate to homogenates. 2, 4-dinitrophenol inhibits esterification of inorganic phosphate without affecting the rate of oxidation.

3. Succinoxidase and cytochrome oxidase are synthesized during development, following in exponential curve that is parallel to the increase in oxygen uptake.

4. The respiratory quotient has a value of 0.75 at the beginning of development and increases sharply during gastrulation. Thereafter, it declines gradually up to hatching.

5. A striking breakdown of glycogen during gastrulation results in high accumulation of lactic acid. This accumulation is disappeared by the beginning of the circulation. The activity of anaerobic glycolysis increases gradually during development.

6. The amount of diphosphopyridine nucleotide is at a constant level during development. Total amounts of thiamin and flavin are rather constant during development, while phosphothiamin and flavinadenine dinucleotide are synthesized after gastrulation.

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ON THE CARBOHYDRATE COMPONENT OF THE JELLY COAT AND RELATED SUBSTANCES OF EGGS FROM JAPANESE SEA URCHINS[1)]

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The significance of polysaccharide in the fertilization of sea urchin eggs has been accentuated by extensive studies on the jelly coat substance. Chemical analyses indicated that the jelly coat is composed of a mucopolysaccharide with sulfuric acid residues (VASSEUR 1948, TYLER 1949). Moreover, it has been shown in some species of European sea urchins that the carbohydrate component of the jelly coat has a species-specific composition (VASSEUR and IMMERS 1949). It may be, therefore, of interest to investigate carbohydrate compositions of the jelly coats of Japanese sea urchins in order to see whether or not they differ among species.

Furthermore, the possibility has been considered that polysaccharides are of importance for the structural changes of the egg. The separation of the fertilization membrane and the formation of the hyaline layer are remarkably visible changes at the time of fertilization in the sea urchin egg. According to MOTOMURA (1941) and RUNNSTRÖM (1947), the fertilization membrane is formed from the vitelline membrane which covers the surface of the unfertilized egg and the substance of the cortical granules which present below the protoplasmic surface in the unfertilized egg. Recently MONNÉ and SLAUTTERBACK (1950) demonstrated that the cortical granules and the hyaline layer exhibit similar staining properties to those of the jelly coat and postulated that they also contain mucopolysaccharide. However, the exact chemical nature of these substances is not known. The present paper deals with the preliminary experiments on the isolation and carbohydrate component of these substances.

This work was carried out in part at the Sugashima Marine Biological Station.

EXPERIMENTAL

Material. The jelly coat substance was prepared from the eggs of some sea urchin species found on the Pacific coast of Japan, *viz.* ***Hemicentrotus pulcherrimus, Pseudocentrotus depressus*** and ***Heliocidaris crassispina.*** Both cortical granule and hyaline layer substances were prepared from *Hemicentrotus*

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eggs, because this species was abundunt in the bay near the station.

Paper partition chromatography. In every case, the preparation was hydrolyzed for 3~6 hours with 2 ml. of 30 per cent KOH in a sealed tube at 98° C. Then the solution was hydrolyzed with 2 N sulfuric acid for 6 hours in a sealed tube at 98° C. The solution was neutralized with barium carbonate and filtered. The filtrate was concentrated under reduced pressure to a syrup. A portion of the syrup was dissolved in water and a small portion of it was separated on the chromatogram. Ascending chromatograms were run on Toyo no. 50 papers. The developers used were *n*-butanol-acetic acid-water and phenol-ammonia. Galactose, glucose, mannose, arabinose, fucose and galactouronic acid were used as markers. The spots were colored with aniline hydrogen phthalate (PARTRIDGE 1948, 1949).

Jelly coat substance. The jelly solution was prepared from heavy egg suspensions subjected to treatment with sea water of pH about 4.5. The jelly coat substance went into solution at this pH. Eggs were centrifuged off and the supernatant jelly solution was precipitated with ammonium sulphate at 50 per cent saturation. The precipitate was dialyzed, after the addition of small amount of water, against running tap water and finally distilled water until free from salts of sea water and ammonium sulphate. Afterwards the samples were dried in vacuum.

The hydrolyzed preparation of the jelly coat substances of *Hemicentrotus pulcherrimus, Pseudocentrotus depressus* or *Heliocidaris crassispina* showed a single spot on the paper chromatogram. The only sugar identified so far was fucose. There was no evidence of the presence of appreciable amounts of other sugars in these preparations. However, if the hydrolysis was incomplete, a different weak spot was formed, having the Rf value of 0.13 (*n*-butanol-acetic acid-water) or 0.05 (phenol-ammonia). This may correspond to the oligosaccharide of fucose, because it disappeared after further hydrolysis.

Cortical granule substance. The cortical granule substance was prepared in the following way. The jelly coat of unfertilized eggs was previously removed by repeated washings with acid sea water. Then the eggs were washed with normal sea water and finally treated with 1 M urea solution for 5 minutes. As shown by MOTOMURA (1941, 1947), the cortical granules went into this solution. The supernatant was collected by slow centrifuging and filtered. The clear colorless filtrate was precipitated with ammonium sulphate at 50 per cent saturation. The substance could be precipitated with either Janus green or alcohol and ether. The precipitate was dialyzed against running tap water and distilled water until free from urea and ammonium sulphate. During this process, the substance dissolved and a very viscous solution was obtained. Finally this concentrated solution was frozen-dried. This material was hydrolyzed as above and the paper chromatographic analysis was run. The chromatograms of this substance showed the presence of fucose only.

It should be noticed that the vitelline membrane is also dissolved in 1 M urea solution (MOORE 1930, MOTOMURA 1941). Though the dissolution of the vitelline membrane was avoided by adding a small amount of sea water to the

urea solution, there is a possibility of slight contamination with the substance derived from the vitelline membrane. It is, therefore, more convenient to use eggs from which the vitelline membrane has previously been removed by trypsin digestion. The trypsin-treated eggs, however, showed a tendency of cytolysis in the urea solution with the result that the contamination with cytoplasmic inclusions was increased. In both cases, no difference could be observed with respect to the carbohydrate component. A more precise study on the isolation and chemical properties of this substance is in progress. In the present paper, the substance prepared as above is tentatively called the cortical granule substance.

Hyaline layer substance. The hyaline layer substance was prepared from *Hemicentrotus* eggs. Eggs were fertilized and the fertilization membrane was removed immediately after its separation by filtering with bolting silk (LINDAHL and LUNDIN 1948). The denuded eggs were kept for 15 minutes in rather dilute population, and then concentrated by sedimentation under gravity. After repeated washings, eggs were treated for 5 minutes with 5/9 M NaCl or 1 M urea solution and centrifuged off. The hyaline layer substance was completely dissolved in urea solution, but some of the eggs were often cytolyzed. For the preparative work, the clear colorless supernatant fluid was used in order to avoid contamination with cytoplasmic inclusions. To the supernatant fluid, ammonium sulphate was added at 30 per cent saturation. After standing overnight, the precipitate was sharply centrifuged down, dissolved in distilled water and dialyzed against running tap water and finally distilled water. Thereafter the solution was frozen-dried.

It was found that the hydrolyzate of the hyaline layer substance of *Hemicentrotus pulcherrimus* contains fucose as its single carbohydrate component. Unpublished study indicates that the hyaline layer substance is a lipid-carbohydrate-protein complex. This substance could be precipitated with either Janus green or alcohol and ether.

DISCUSSION

It has been considered that species specificity of the fertilization may depend on the carbohydrate component of the jelly coat or those of the egg surface. VASSEUR and IMMERS (1949) showed that chemical differences exist between the jelly coats of some species. In *Echinus esculentus* only galactose and in *Echinocardium cordatum* only fucose residues were found. The jelly of *Strongylocentrotus droebachinensis* consists of fucose and galactose and that of *Paracentrotus lividus* of fucose and glucose. TYLER (1949) described that the jelly coat of *Strongylocentrotus purpuratus* is composed of galactose. BISHOP and METZ (1952) also reported that the hydrolyzate of the jelly of the sand-dollar, *Echinarachnius parma,* contains fructose as its single carbohydrate component. In three species of Japanese sea urchins, *viz. Hemicentrotus pulcherrimus, Pseudocentrotus depressus* and *Heliocidaris crassispina,* it was found that the jelly coats were composed of only fucose. This seems to be in contradiction to the statement of VASSEUR and IMMERS (1949) that the carbohydrate component of

the jelly coat has a genus specific, perhaps even species specific composition. However, it seems likely that the jelly coats of these species contain a similar, but not identical component.

In addition to the present results, there is evidence from the experiments on the cross-fertilization. ONODA (1937) studied the cross-fertilization between various species of Japanese sea urchins and showed that the best result was obtained in the reciprocal combination between *Hemicentrotus pulcherrimus* and *Heliocidaris crassispina*. OKA (1952) has also reported that the cross-fertilization is very successful between *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. These results suggest that the jelly coats of these species have similar properties.

The source of the jelly coat substance seems to be of particular interest. Recent analyses by IMMERS (1952) demonstrated that the jelly-less egg of *Paracentrotus lividus* does not contain fucose. Neverthless, the jelly coat of this species contains fucose as its dominating carbohydrate component. This means that the jelly coat is not secreted by the egg. Our results indicate, however, that the egg substances, such as the cortical granule and the hyaline layer substances from the eggs of *Hemicentrotus pulcherrimus*, also contain fucose. It can be assumed that these substances may have a similar composition to those of the jelly coat substances. Furthermore, the histochemical studies carried by MONNÉ and SLAUTTERBACK (1950) support this view, since these authors showed that acid polysaccharides are important constituents of the hyaline layer and the cortical granules.

The question arises whether polysaccharides are uniform in these structures. They may be complex of one fundamental unit, or may vary according to the structures. Although the answer have not yet been made to this problem, there is one further evidence from experiments with other respect. Recently MOTOMURA (1950, 1953) has reported that the fertilized eggs can secrete the sperm agglutinin which is different from the jelly coat substance. The secretion of this substance takes place during first ten minutes after fertilization. This suggests that mucopolysaccharides similar to the jelly coat substance exist in the inner layer of the egg and they are released upon fertilization. In summary, it would appear that a close correlation may be present between the jelly coat, the cortical granules and the hyaline layer.

SUMMARY

1. The carbohydrate component of the jelly coats of the Japanese sea urchins, *viz.* *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus* and *Heliocidaris crassispina*, was analyzed by means of a paper partition chromatography and the presence of fucose was demonstrated.

2. The substances of the cortical granule and the hyaline layer were isolated from the eggs of *Hemicentrotus pulcherrimus* and it was found that their hydrolyzate contained fucose as its single carbohydrate component.

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HIRATA, Chemical Institute of Nagoya University, for their valuable criticism and suggestion concerning the experiments.

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A METHOD OF COMPUTATION OF THE SURFACE AREA OF THE CELL*

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In 1940 MOTOMURA calculated the surface area of sea urchin blastomeres by considering that a blastomere is composed of a series of trapezoids. He states that the error involved in this procedure is 5%. However, the calculation becomes much simpler and more accurate by thinking that a blastomere consists of series of zones and segments of spheres. A zone of a sphere is a disc-shaped section of a sphere between two parallel planes and a segment of a sphere is a portion of a sphere cut off by a plane (Fig. 1).

The formula for the area of the curved surface of zones and segments of spheres is πDH; D being the diameter of the original sphere and H being the height of the segment or the zone (see Fig. 1). Therefore, for the computation of the surface area of a blastomere, the shape of which is not necessarily spherical, it is necessary to divide the blastomere by parallel planes into as many sections as are necessary to permit the assumption that the separate sections are zones or segments of spheres of different diameters.

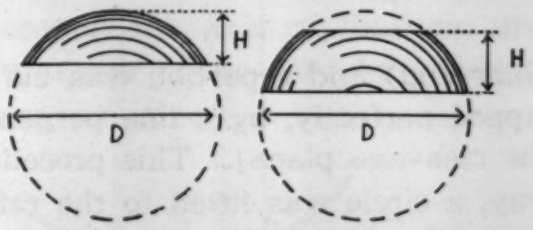

Fig. 1. A segment of a sphere (*left*) and a zone of a sphere (*right*).

For the application of this principle to the concrete case of the sea urchin blastomere, however, one is spared from dealing with the problem in three dimensions, thanks to another condition, *i.e.*, that the cross sections of the blastomeres cut by planes parallel to the cleavage plane can be considered as circular. As a result, given two-dimensional drawings of the blastomeres in the plane of the spindle, the other dimension will be taken care of by the circularity of the cross sections. The procedure was as follows (see Fig. 2): 1) Camera lucida drawings of blastomeres were made at consecutive stages of cleavage. 2) Using one of these, the axis of rotation was found by folding the paper in such a way that the upper and lower halves of the drawing overlapped as much as possible. The line of folding was taken as the axis. 3) A circle was found which would circumscribe one end of the blastomere (either the furrow or the

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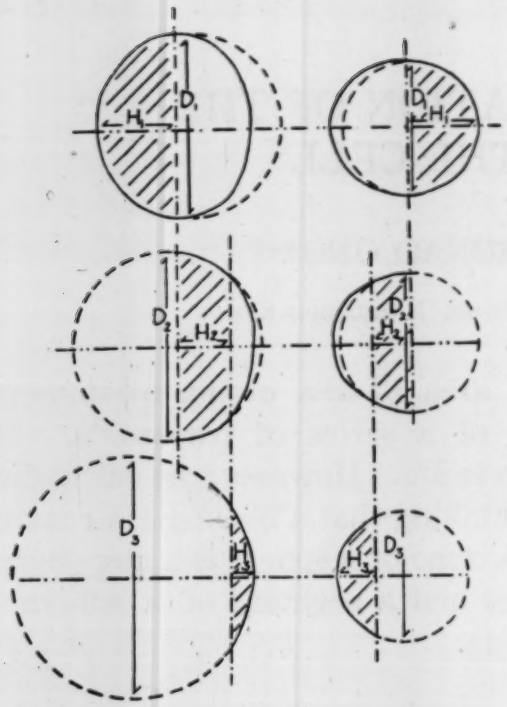

Fig. 2. Procedure for the computation of the surface area of the blastomeres. The process is divided into three steps (from the top to the bottom), the shaded parts being considered separately. The shape of the cell is represented by a real line at the top of the figure. The left one is a blastomere of the 2-cell stage; the right one is that of the 4-cell stage. Horizontal lines (—··—··—) are axes of rotation. *D*'s are the diameters of the circumscribing circles and *H*'s are the heights of the segments and the zones of spheres. $\Sigma\pi DH$ gives the total area of the cell.

polar end) and a portion was cut off where the contour and the circle overlapped perfectly, by a line perpendicular to the axis of rotation (or parallel to the cleavage plane). This procedure gives a zone of the sphere. In a similar way, a circle was fitted to the remaining portion which would give a segment of a sphere of a different diameter. This process was repeated until the entire blastomere surface had been covered. 4) The diameters of the circumscribing circles (D_1, D_2, D_3, . . .) and the heights of the sections (the distances between adjacent parallel lines, H_1, H_2, H_3, . . .) were measured. The total area of the blastomere was then $\Sigma\pi DH$.

Ordinarily, dividing a blastomere into three or four sections suffices for the circumscription of its entire contour. When the blastomere contour is exceptionally irregular, although a little more complicated procedure might be required, the authors have not so far encountered any case which could not be handled according to this principle. (see Fig. 3) As was mentioned, the only assumption involved in this procedure is the circularity

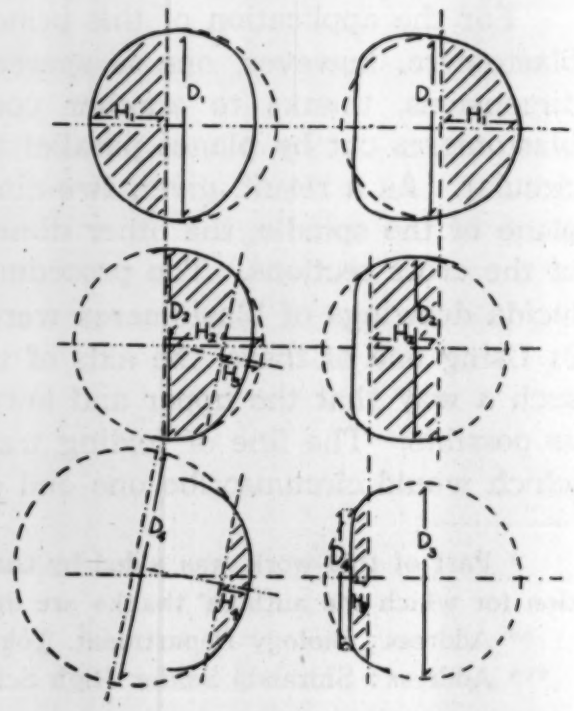

Fig. 3. The cell on the left side is asymmetric in reference to the original rotational axis. This case can be handled by introducing the second axis. The total surface area is given as $\{D_1H_1 + D_2(H_2 - H_3) + D_4H_4\}\pi$.

The right hand cell is flattened on one end. The surface area can be obtained as $\{D_1H_1 + D_2H_2 + D_3H_3 + D_4^2/4\}\pi$.

of the cross section of a blastomere by planes parallel to the cleavage plane, and this assumption is found to be practically true by actual observation.

Regional Changes in the Surface Area of the Dividing Blastomere

Our group at the Misaki Marine Biological Station has reported on regional shrinkage and expansion of the blastomere surface by measuring the distances between two kaolin particles attached to the blastomere surface (Dan, Yanagita and Sugiyama '37; Dan, Dan and Yanagita '38; Dan and Dan '40). Naturally measurements of this kind give linear shrinkage or expansion of the surface along the circumference of the largest optical section of the cell. Numerous past records indicate that such particle distances undergo regionally characteristic changes according to their topographical positions in reference to the cleavage furrow (or to the spindle axis). Four different regions can be recognized in the eggs of the sea urchin, *Mespilia globulus*, which the author calls the furrow-, sub-furrow-, sub-polar- and polar-regions (Fig. 4). Since these regions are parallel zones of uniform widths girdling the cell around the axis of rotation, areal changes of the surface can be calculated from the linear data by using the above-discussed method.

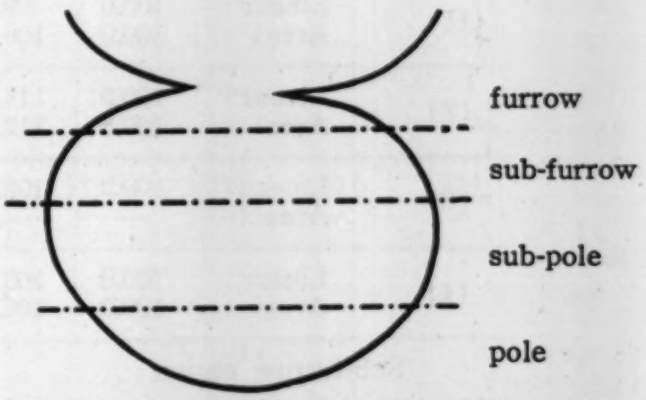

Fig. 4. Four regions as they are defined by the movement of the cell surface.

However, the procedure must be modified to some extent when it is applied to the polar region of the cell. By definition, the polar region is a thin cap covering the spindle pole area. Consequently, two kaolin particles attached to the region may be situated more or less symmetrically on two sides of the rotational axis (see Fig. 4). As a result, two lines passing through the particles would at most give a very thin segment of a sphere or sometimes even coincide in a single line. Under such circumstances, the calculation of the area becomes extremely inaccurate. This inconvenience can be obviated by taking only one particle and obtaining the surface area of the zone distal to the particle. But if this method is adopted, another difficulty is encountered. It has been noticed for a long time that the polar surface is pulled to one side or to the other around the spindle axis as the result of unbalanced strains developed during the course of cleavage. This side shift in position does not interfere with linear measurements since both particles move together, while it invalidates the area calculation using one particle. An error involved in the determination of the direction of the rotational axis also offsets the calculation in the same way. In short, the difficulty of the calculation of the polar region lies in the fact that lines along the circumference connecting any two particles run perpendicularly to the rotation axis, while in other regions, they are more or less parallel to it.

For the above reasons, the curves of the polar region are very irregular. For the other three regions, this difficulty does not arise and the curves are, in fact, far more regular. Data are given in Table 1.

In Figures 5, 8, 9 and 10, curves of areal changes are shown side by side

Table 1. Data of the linear and the areal changes of the initial spherical condition. Figures with

Polar region								
(1)	Linear	100.0	103.3	103.9	108.5	107.2	117.0	111.8
	Areal	100.0	82.0	98.2	83.9	99.4	102.7	96.8
(2)	Linear	100.0	102.0	115.3	117.3	120.7	109.3	114.0
	Areal	100.0	126.4	98.9	103.1	123.3	90.8	107.5
(3)	Linear*	100.0	102.0	106.5	107.2	112.8	116.1	120.9
	Areal	100.0	109.4	87.2	100.4	127.9	145.2	142.4
(4)	Linear	100.0	119.9	119.3	124.5	128.0	123.9	107.6
	Areal	100.0	98.5	87.6	115.8	106.7	97.6	73.5
Sub-polar region								
(1)	Linear	100.0	99.0	111.0	113.1	125.1	122.0	120.4
	Areal	100.0	106.1	123.2	130.3	141.4	145.6	132.1
(2)	Linear*	100.0	115.0	123.0	126.0	123.0	119.0	114.0
	Areal	100.0	117.5	140.1	162.2	158.0	158.3	132.7
(3)	Linear*	100.0	102.0	100.7	103.3	105.0	115.8	112.4
	Areal	—	—	—	100.0	135.0	176.0	—
(4)	Linear	100.0	105.8	115.3	115.8	119.5	126.3	130.0
	Areal	100.0	106.4	117.4	118.1	117.0	131.0	135.1
Sub-furrow region								
(1)	Linear*	100.0	97.3	118.1	118.1	146.0	156.1	136.6
	Areal	100.0	101.7	127.6	115.7	138.8	141.1	124.6
(2)	Linear*	100.0	100.0	121.0	143.0	161.0	161.0	146.0
	Areal	100.0	123.6	153.3	171.0	186.1	179.7	162.3
(3)	Linear*	100.0	101.5	101.5	98.4	100.0	113.0	114.7
	Areal	100.0	—	—	101.7	114.0	120.8	113.7
(4)	Linear*	100.0	103.0	105.0	122.0	144.0	157.0	150.0
	Areal	100.0	90.5	93.4	97.8	106.2	116.0	106.4
Furrow region								
(1)	Linear*	100.0	93.0	93.0	93.0	75.3	76.6	80.0
	Areal	100.0	—	86.1	71.8	53.6	—	—
(2)	Linear*	100.0	105.0	76.2	86.0	88.1	100.0	125.3
	Areal	100.0	—	—	68.5	66.7	72.5	81.6
(3)	Linear*	100.0	109.9	117.0	95.1	114.6	126.5	129.2
	Areal	100.0	115.0	86.0	73.6	—	58.4	—

with those of linear changes among which the polar region is tentatively included (Fig. 5). In order to supplement this defect of the polar data, surface-view observations were also made. If colored particles such as carmine or carbon particles are made to adhere to the egg, those on the surface can be seen as well

surface in various regions expressed in percentages of the
asterisks are previously published data.

116.3 128.0	109.8 83.9	108.5 87.5							
121.0 167.2	120.4 164.7	119.0 164.7	116.6 139.0	113.1 131.1					
122.9 86.1									
114.7 114.7									
123.0 —	121.4 —	121.4 198.1	127.2 —	128.0 —	122.9 176.7	115.7 —	122.1 186.8	120.0 —	117.1 —
126.8 128.3	118.9 117.0	112.1 103.0							
131.7 127.0									
142.7 125.2	144.1 121.4	160.7 143.7	163.9 132.9	145.8 —					
81.0 —	88.9 99.9	129.0 —	149.0 —	182.0 96.0	172.0 98.5				
152.1 83.2	159.0 87.3	208.0 164.0	252.5 158.8	252 5 154.1					
195.0 92.3	244.0 142.0	251.0 —							

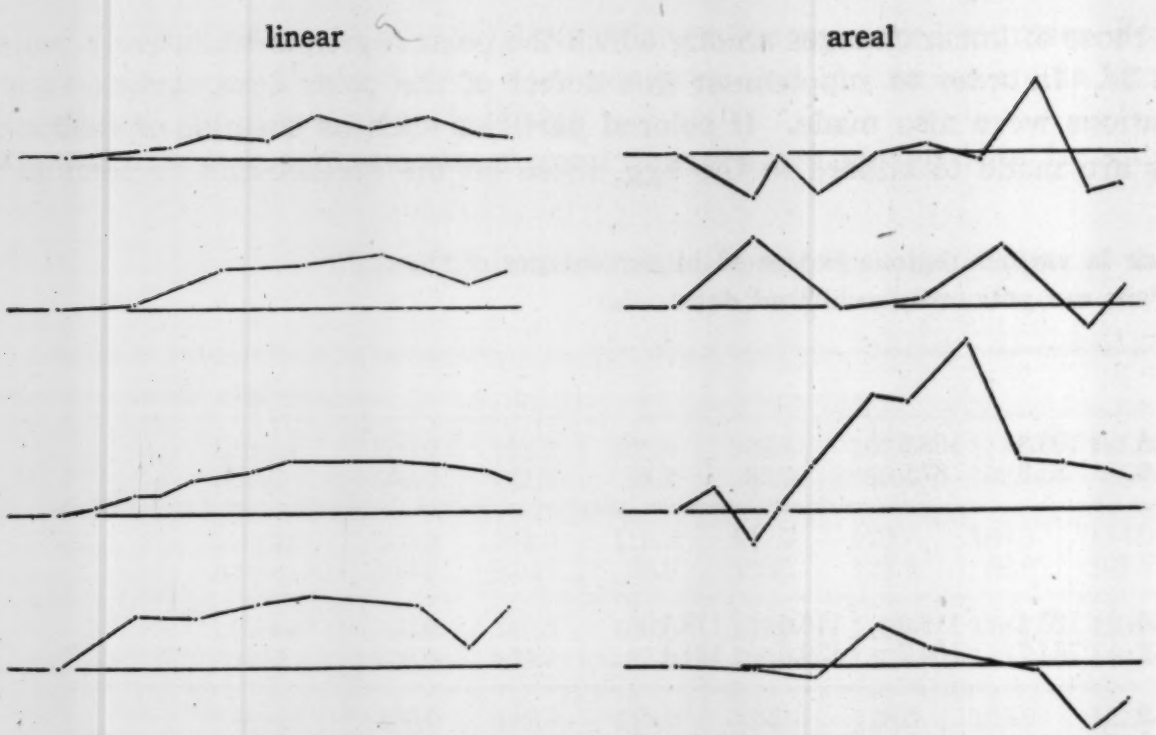

Fig. 5. Comparison of the linear and the areal changes of the surface of the polar region. The areal curves show a tendency of expansion but they are rather irregular. For the reason, see the text.

as those on the circumference of the largest optical section. If several of them are selected and the change in the shape of the constellation formed by the particles is followed through the cleavage process, the surface distortion can be studied more easily. Figures 6 and 7 illustrate the movement of the polar surface. Although such records give at best only qualitative information, the fact that attached particles move away from the pole during cleavage must indicate an expansion of the polar surface. Considering the shift of the particles here shown, as well as the linear measurements obtained earlier, and also taking into consideration the behavior of the adjacent sub-polar region (see below), one may be justified in thinking that the polar region must be expanding in area.

The curves for the sub-polar region are much like the linear curves in shape (Fig. 8). Moreover, the maxima of the two groups quite frequently coincide in time too. However, it does not seem possible to ascribe to this region a characteristic magnitude of the expansion as was done for the linear data. The linear curves on the left hand side of Fig. 8 are arranged in the order of greater stretching, but the areal curves corresponding to them do not show a correlative tendency.

For the sub-furrow and the furrow regions, the two sets of curves differ rather widely in shape. Starting from the sub-furrow region (Fig. 9), the surface behavior of this region is linearly characterized by a greater stretching than the preceding polar regions. Although there is a variation in behavior, it might be possible to say, if anything, that the areal curves are lower than those of the sub-polar region. The last areal curve is of some interest. The course of this curve is quite different from the others, but as will be shown in the

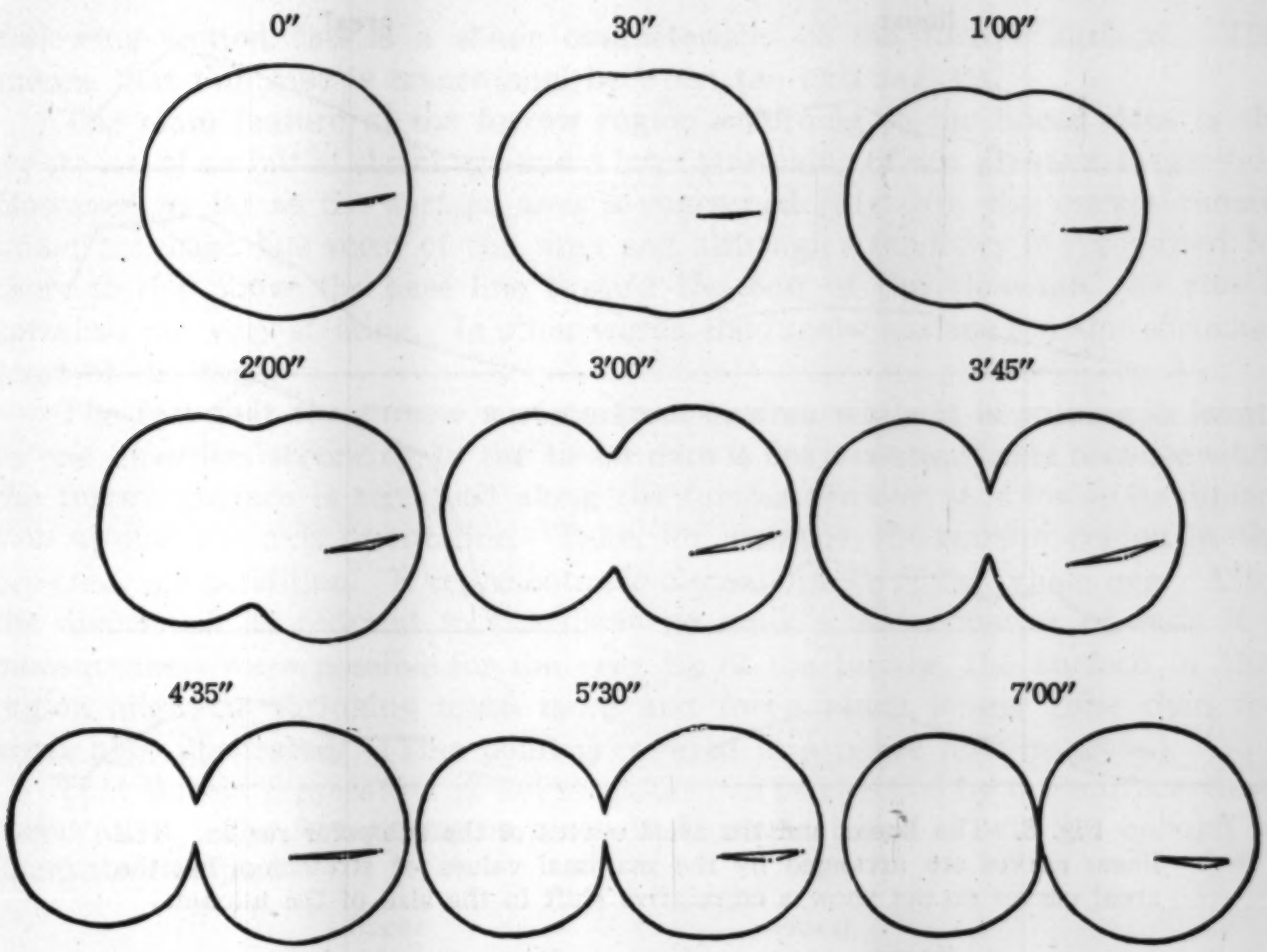

Fig. 6. Records of surface-view-observation, showing the expansion of the polar area.

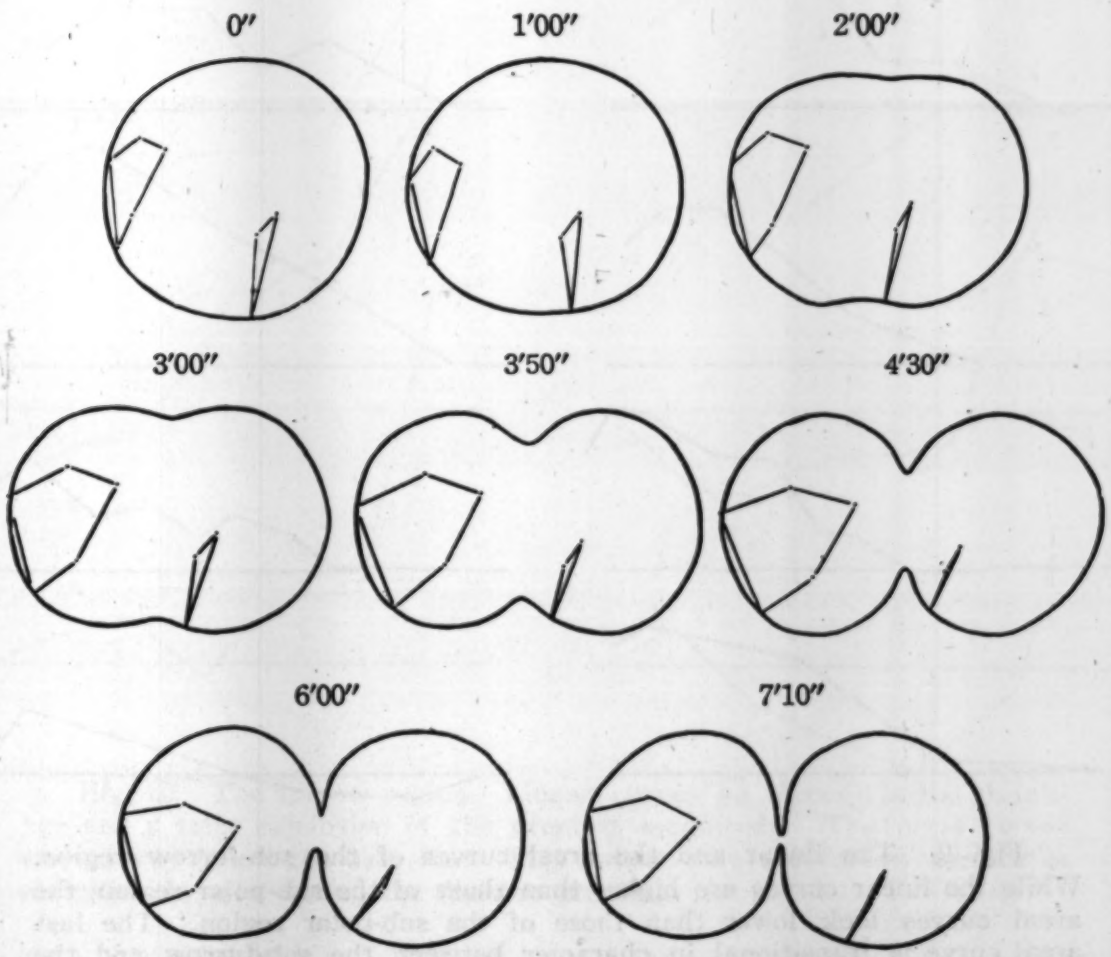

Fig. 7. The same as Fig. 6. Note particularly the pentagon on the left hand side.

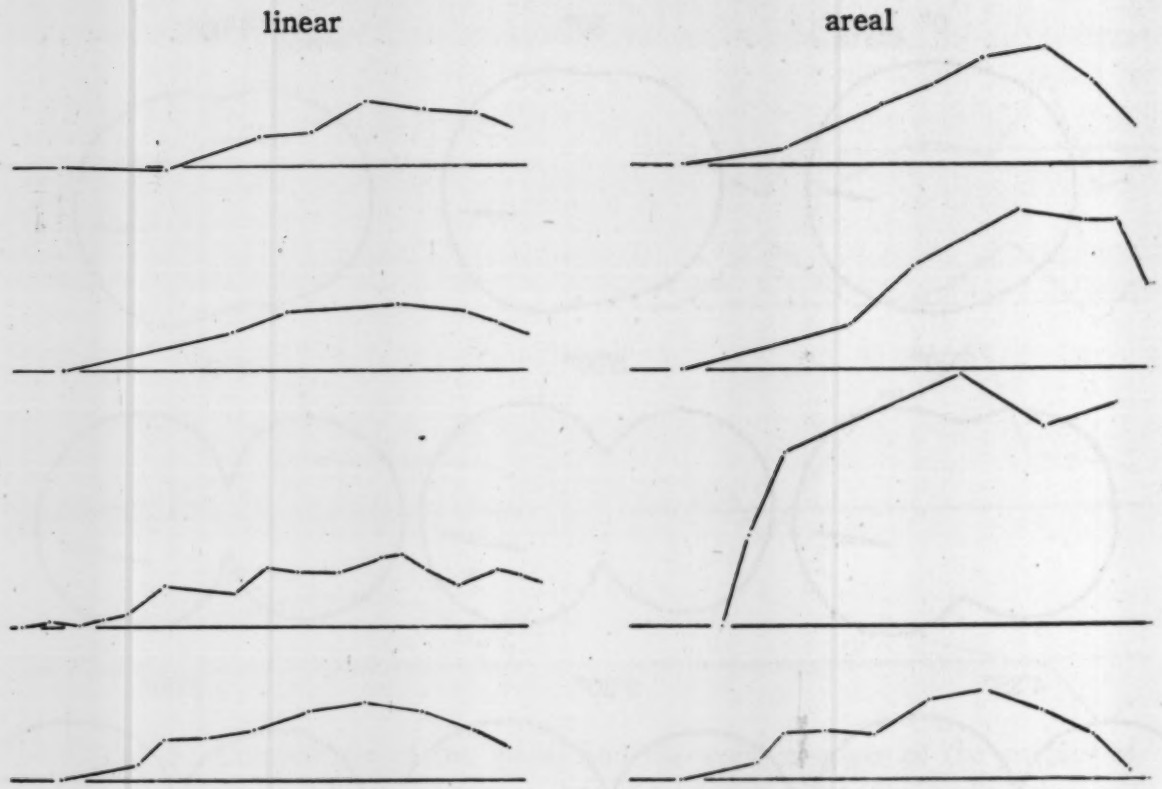

Fig. 8. The linear and the areal curves of the sub-polar region. The linear curves are arranged by the maximal values of stretching, but the areal curves do not show a correlative shift in the size of the maxima.

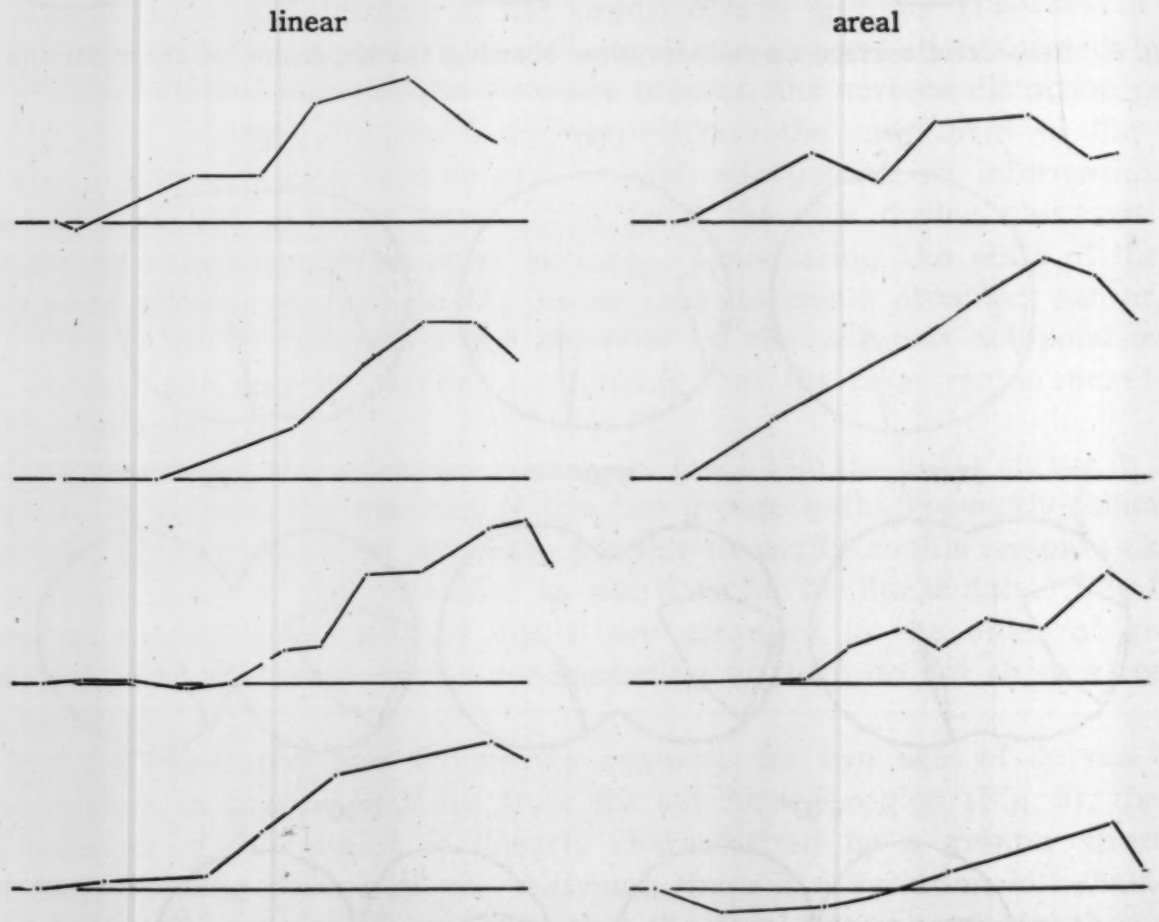

Fig. 9. The linear and the areal curves of the sub-furrow region. While the linear curves are higher than those of the sub-polar region, the areal curves look lower than those of the sub-polar region. The last areal curve is transitional in character between the sub-furrow and the furrow region (see Fig. 10).

following section, this is a shape characteristic of the furrow surface. This means that this case is transitional between the two regions.

The main feature of the furrow region according to the linear data is the existence of an initial shrinkage and a later stretching of the greatest magnitude. However, as far as the surface area is concerned (Fig. 10), the curves remain under the base line most of the time and although a tendency is recognised for them to rise above the base line toward the end of the cleavage, the rise is certainly not very striking. In other words, the furrow surface remains shrunken most of the time.

The fact that the furrow surface loses in area while it is gaining in length in one direction according to the linear data is not a contradiction because while the furrow surface is stretched along the furrow contour, it is losing its dimension around the axis of rotation. Take, for instance, the equator region in the pre-cleavage condition. It represents the circumference of the whole egg. After the division, it is reduced to the cleavage stalk. As a matter of fact, if a measurement were possible for the very tip of the furrow, the surface of that region might be shrinking much more and for a much longer time than the areas here illustrated. (This point is covered in a paper now in press.)

That this interpretation is not mistaken can be checked by the surface-view-observation. The few examples given in Figures 11 and 12 will be enough to illustrate this point.

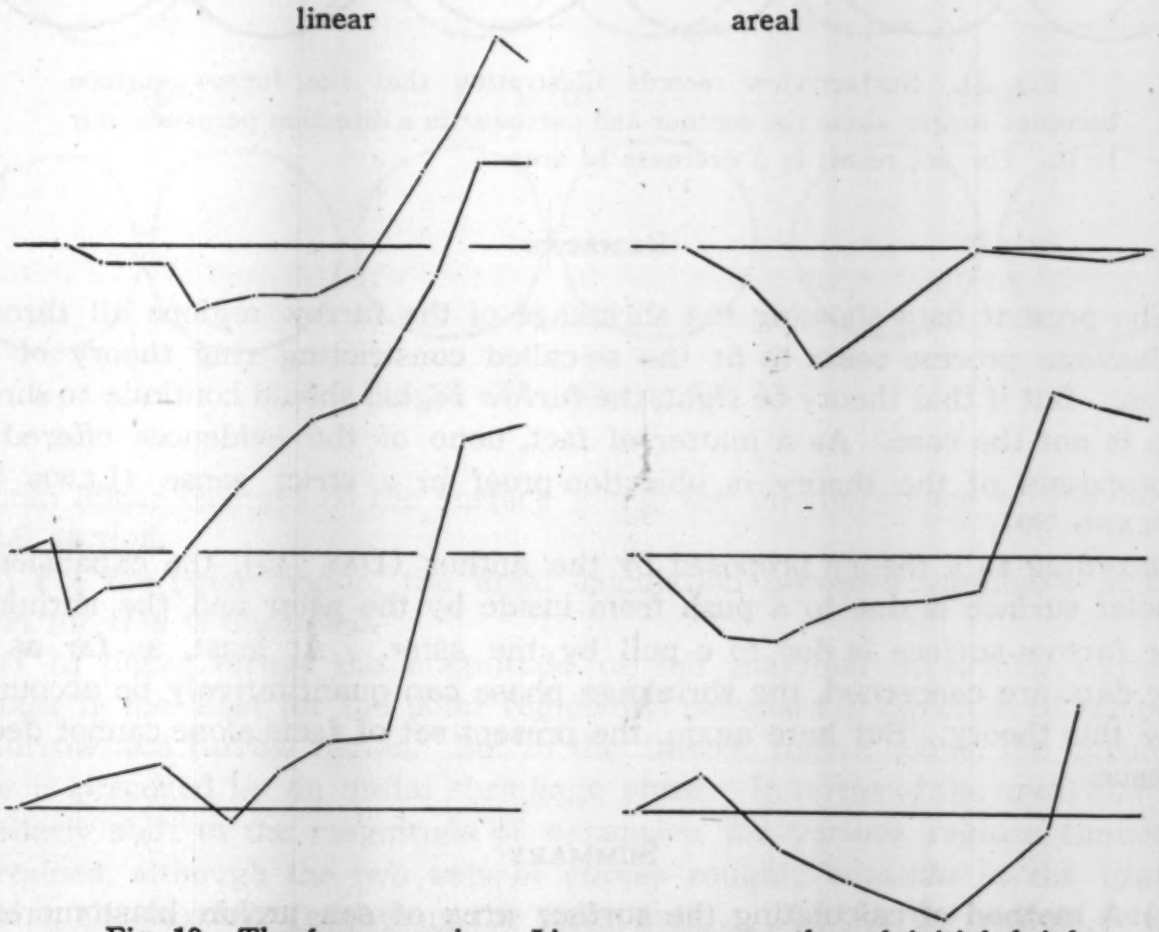

Fig. 10. The furrow region. Linear curves go through initial shrinkage and a later expansion of the greatest magnitude. The areal curves stay mostly below the base line. This means that the furrow surface is shrinking in area during the division process.

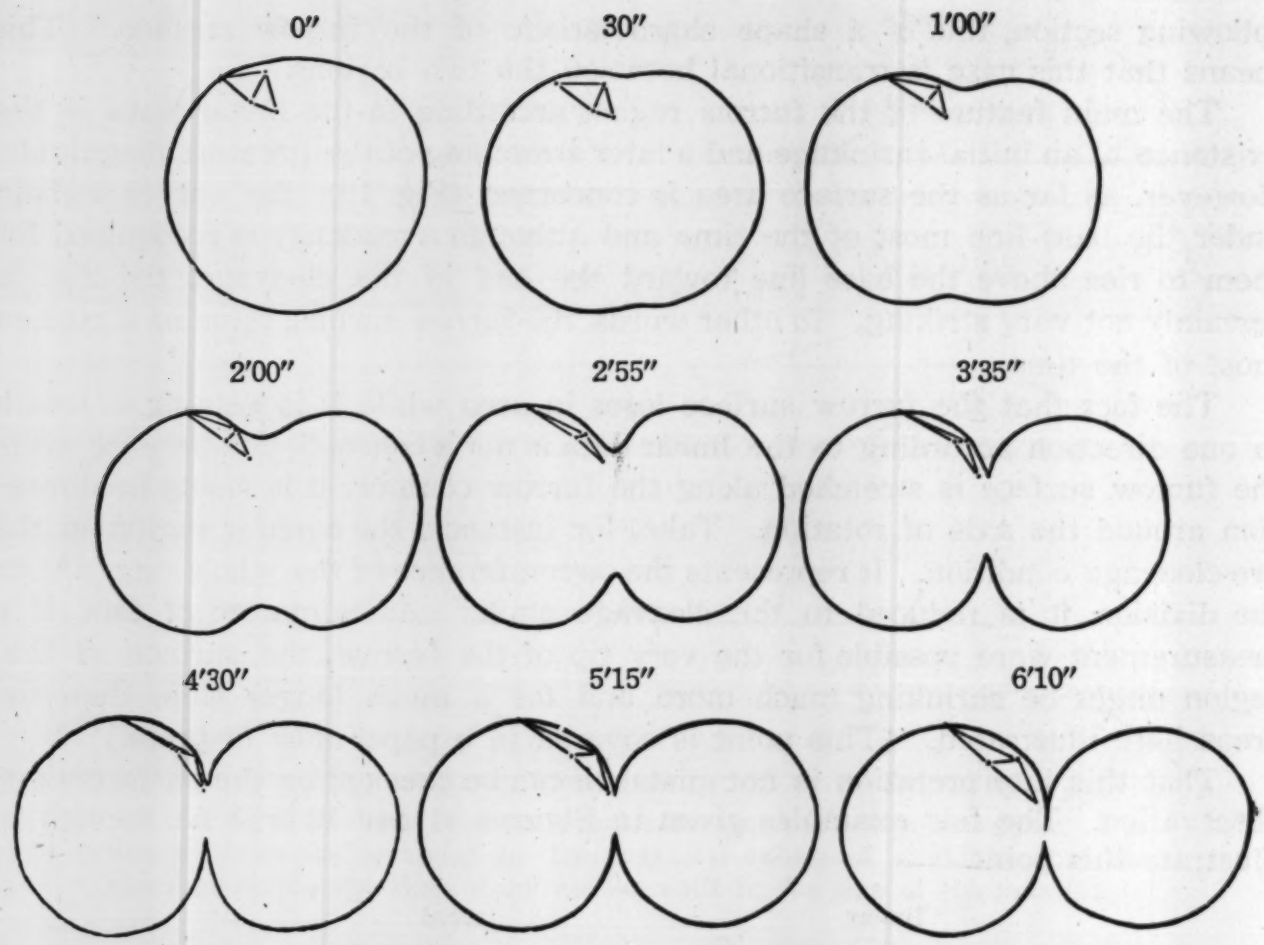

Fig. 11. Surface-view records illustrating that the furrow surface becomes longer along the contour and narrower in a direction perpendicular to it. The net result is a decrease in area.

Remarks

The present data showing the shrinkage of the furrow regions all through the cleavage process seem to fit the so-called constricting ring theory of cell division. But if that theory be right, the furrow region should continue to shrink, which is not the case. As a matter of fact, none of the evidences offered by the proposers of the theory is objection-proof in a strict sense (Lewis '39; Marsland '39).

According to a theory proposed by the author (Dan '43), the expansion of the polar surface is due to a push from inside by the aster and the shrinkage of the furrow surface is due to a pull by the aster. At least, as far as the linear data are concerned, the shrinkage phase can quantitatively be accounted for by this theory. But here again, the present set of facts alone cannot decide the issue.

Summary

1) A method of calculating the surface area of sea urchin blastomeres is described. This method is applicable to other cells of similar shapes.

2) Regional changes of the surface area of the cell through the division cycles are calculated by this method and are compared with previously reported

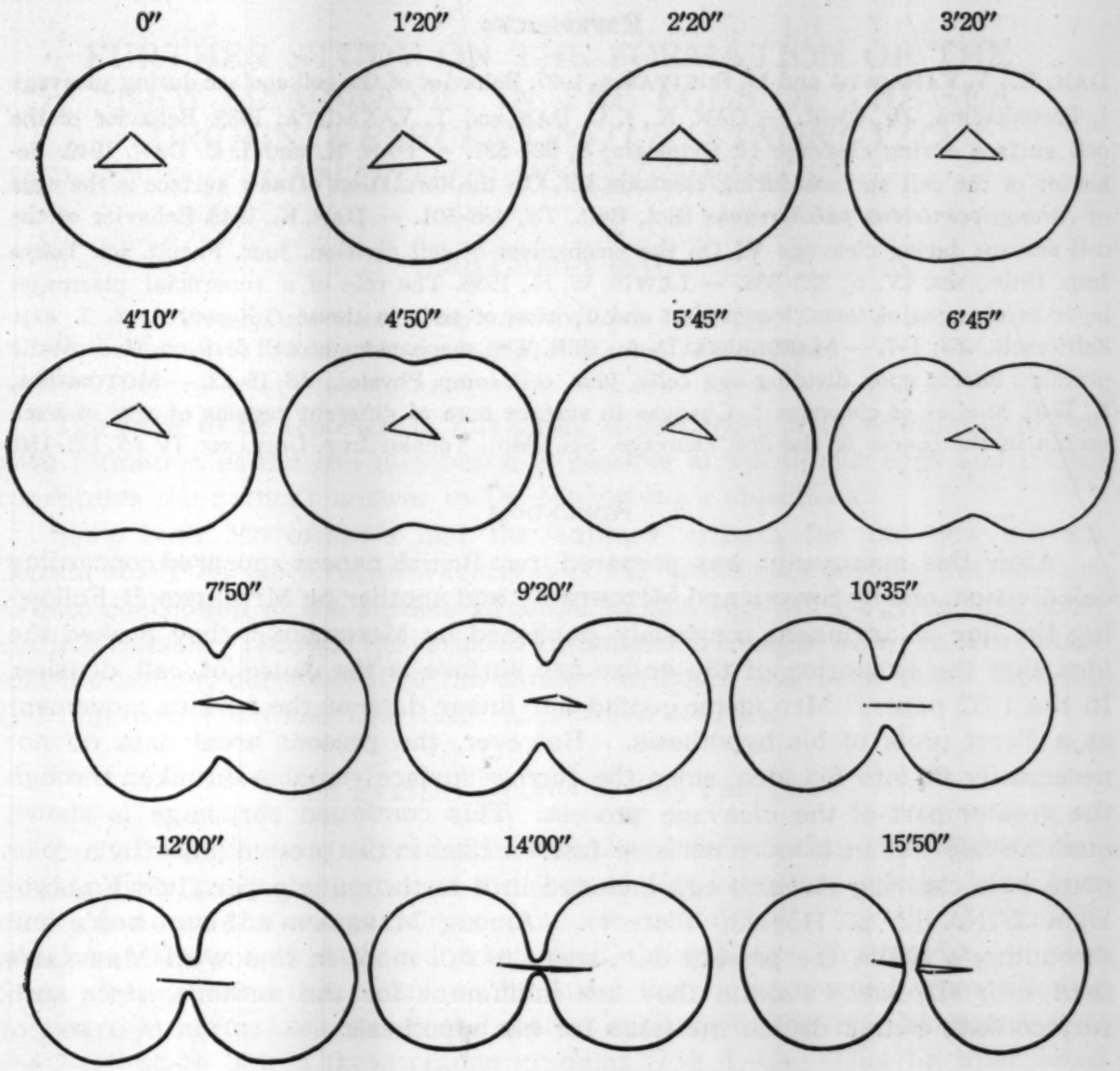

Fig. 12. The same as Fig. 11.

regional linear changes of the surface along the circumference of the largest optical section.

3) For the polar region, the areal calculation becomes unreliable and the reason for this is discussed.

4) In linear terms, the magnitude of the maximal stretching during a cleavage is the least for the polar region and increases in the order of sub-polar, sub-furrow and furrow region. But in the furrow region alone, the expansion phase is preceded by an initial shrinkage phase. In terms of the areal changes, an orderly shift in the magnitude of expansion for various regions cannot be ascertained, although the two sets of curves roughly coincide in the time of reaching the maxima (excluding the polar data). The furrow region remains shrunken practically all through the division process, and in case it expands toward the very end of the cleavage, the magnitude of the expansion is not specially greater than those found in other regions.

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Addendum

After this manuscript was prepared, two British papers appeared concerning cell division, one by SWANN and MITCHISON[1] and another by MITCHISON.[2] Following the line of argument previously published by MITCHISON,[3] they pushed the idea that the expansion of the entire cell surface is the cause of cell division. In the 1952 paper,[3] MITCHISON quoted our linear data of the surface movement as a direct proof of his hypothesis. However, the present areal data do not necessarily fit into his idea, since the furrow surface remains shrunken through the greater part of the cleavage process. This continued shrinkage is shown qualitatively but in a more decisive fashion than in the present paper by a color plate of a cleaving *Arbacia* egg included in a forthcoming paper (1954 Embryologia *2*, No. 12, p. 115-122, Plate 4). Among MARSLAND's, MITCHISON's and the author's views, the present data seem to fall more in line with MARSLAD's than with MITCHISON's while they are indifferent for the author's, since such surface data cannot decide the issue for his hypothesis.

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1) SWANN, M. M. and J. M. MITCHISON, 1954. Cleavage of sea-urchin eggs in colchicine. J. Exp. Biol. *30*, 506-514.

2) MITCHISON, J. M., 1954. Microdissection experiments on sea-urchin eggs at cleavage. *ibid. 30*, 515-524.

3) MITCHISON, J. M., 1952. Cell membranes and cell division. Symposia Soc. Exp. Biol. *6*, 105-127.

FURTHER STUDY ON THE FORMATION OF THE "NEW MEMBRANE" IN THE EGGS OF THE SEA URCHIN, *HEMICENTROTUS (STRONGYLOCENTROTUS) PULCHERRIMUS**

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The work to be reported in this paper is concerned with whether or not *de novo* formation of the cell membrane is possible in sea urchin eggs and it also constitutes the author's answer to Dr. MOTOMURA's objections.

Since both MOTOMURA's and the author's criteria for the new surface depend solely on the structural features of the blastomere surface, the most up-to-date conception of the surface structure of this egg will be given in a schematical fashion. Through the series of arguments exchanged between MOTOMURA and the author, our concept of the surface structure has been much modified and improved (DAN and DAN 1940; MOTOMURA 1941 a).

THE PRESENT STATUS OF THE PROBLEM

In 1935, MOTOMURA succeeded in optically differentiating the pigment-containing granules in the cortex of the eggs of *Hemicentrotus*, taking advantage of the fact that the orange colored granules, containing a carotinoid pigment, stand out sharply as dark red when seen through the Wratten No. 49 blue filter. By means of this technique, he found that in eggs which were allowed to cleave in normal sea water, the contact surfaces of the two blastomeres of the 2-cell stage lacked these orange granules (Fig. 1, *C*). This he interpreted as indicating that the contact surfaces were of a different nature from the rest of the surface and consequently must consist of *de novo* surface.

On the other hand, in eggs kept in a Ca-free medium, the orange granules are evenly distributed over the entire surface of the separated blastomeres. According to MOTOMURA, this means that the new membrane fails to be formed in the Ca-free medium.

In 1936 and 1938, the author and his associates performed studies of the surface behavior during cleavage, using the kaolin method with the eggs of the sand dollar, *Astriclypeus manni*. In a Ca-free medium, kaolin particles adhering to the equatorial region are pulled further and further into the furrow as it deepens, until the end of the cleavage process (Fig. 1, *E*). After the cleavage

* This research was aided by the Research Expenditure of the Ministry of Education for which the author's thanks are due.

is completed, however, the particles which have been on the furrow surface are gradually pushed out of the furrow (Fig. 1, *F*). If such movement of the particles is looked upon as indicating the appearance of a new surface around the cleavage stalk, the observed fact can be interpreted to mean that the *de novo* surface is formed even within a Ca-free medium. The new surface is not formed before the end of cleavage, but only after the completion of the cleavage process. In 1940, DAN and DAN combined the filter method and the kaolin method and arrived at the same conclusion for the present material (*Hemicentrotus pulcherrimus*).

The first controversy between MOTOMURA and the author, as to whether the *de novo* surface is formed during the process of cleavage or after its completion, is continuous with another question, whether the formation of the new surface itself can be an essential part of the cleavage process or is a post-cleavage phenomenon which has no direct connection with the division mechanism. The second controversy, as to whether or not the *de novo* surface appears only in the presence of Ca-ions, is a purely technical problem.

DIFFERENCES IN TECHNIQUE EMPLOYED

In a paper published in 1941(b), MOTOMURA attacked the author's conclusion on the basis of possible calcium contamination in the author's experiments. He states that fewer than 10 washings with a Ca-free solution cannot strictly be considered sufficient, because of the diffusion of calcium ions from within the eggs. He believes that 15 washings are absolutely necessary; following such treatment, the pigment granules are distributed evenly and the new surface fails to make its appearance. Under this condition, the blastomeres fall completely apart and frequently become amoeboid. In this work, he adds the presence or absence of the surface halo (Strahlensaum of HERBST '00, p. 456) in a Ca-free medium as a criterion for the diagnosis of new surface. He further maintains that the fact that the blastomeres are in contact in some of our illustrations (DAN and DAN '40, Pl. I, Fig. *E* and Textfig. 2) is a sign of calcium contamination in our solutions.

In order to obtain a really Ca-free condition, thorough washing is naturally desirable. On the other hand, however, in order to insure as nearly normal development as possible, handling should be kept at a minimum, since repeated washing inevitably introduces factors such as mechanical injury and a consequent possible liquefaction of thyxotropic gels, or even cytolysis. To which of the above precautions an investigator will attach importance is purely a matter of his subjective preference. MOTOMURA pursued the first line, while the author more or less followed the second line. Some differences in their experimental procedures arising from the difference in their attitudes which have close bearing on the present paper will be compared. In the following summary, *M* stands for MOTOMURA and *D* for DAN.

Removal of the fertilization membrane. *M*: Eggs are shaken in a test tube. *D*: Eggs are sucked into a fine pipette after the addition of 9× volume of Ca-free solution.

Ca-free solution. *M*: HERBST's Ca-free solution containing SO_4 ions. *D*: Chloride mixture containing NaCl, KCl, $MgCl_2$ and $NaHCO_3$.

Washing process. *M*: Egg suspension is mixed with 9× volume of Ca-free solution and eggs are made to settle and the supernatant is changed, so that after n washings, the initial concentration c of calcium ions will become $c \times 10^{-n}$. Since the initial concentration c of the sea water is approximately 10^{-2} M, the final concentration of calcium ions after n washings can practically be taken as $10^{-(n+2)}$ M. *D*: Eggs are collected by gentle rotation at the center of a large watch glass of 9 cm. in diameter holding 25 ml. and the solution is changed several times as thoroughly as possible. This gentle way of handling may admittedly be rather inefficient as a washing process, although the author believes it to be sufficiently thorough for practical purposes.

Conditions for observation. *M*: Eggs are kept in a watch glass. *D*: Eggs are sealed in a depression slide so as to avoid mechanical disturbances.

Under this condition, the blastomeres, after clearly seperating on division, come back into contact again when the stalk dissolves. (See also the following paper.) If eggs are left in an open dish and the dish is moved, even from the table to the microscope stage, the blastomeres fall completely apart. For this reason, MOTOMURA's above-mentioned objection is considered invalid.

Criteria for the judgement of new surface. *M*: Absence of the orange granules (or by using the filter, the absence of the red color in the cortex) and the absence of the surface halo. *D*: Absence of the extra-granular zone, which is the zone containing the cortical granules in the unfertilized egg and

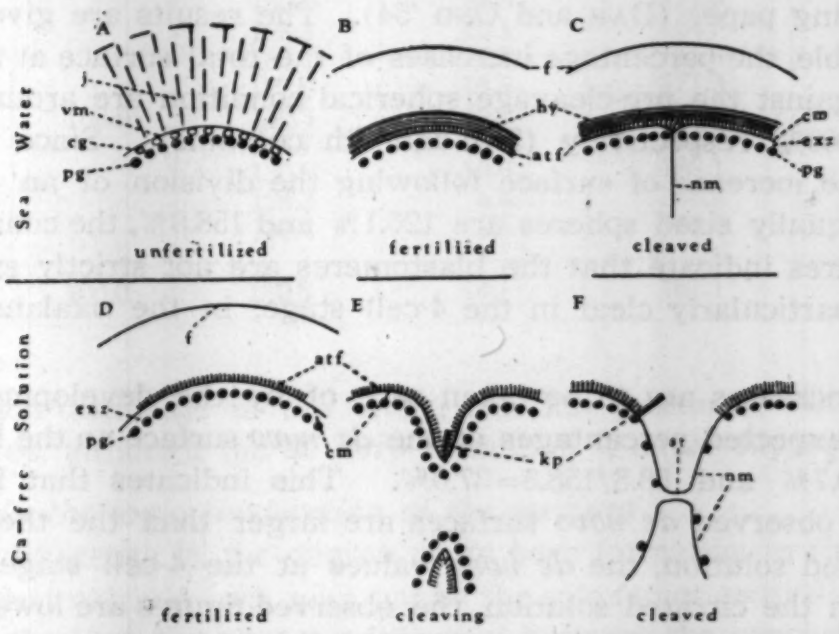


Fig. 1. Schematic representation of the surface structure and its changes in normal sea water and Ca-free medium and also in fertilization and cleavage of *Hemicentrotus* eggs. *atf*: attachment fibers (surface halo). *cg*: cortical granule. *cm*: cell membrane. *exz*: extra-granular zone. *f*: fertilization membrane. *hy*: hyaline layer. *j*: jelly hull. *kp*: kaolin particle. *nm*: new membrane. *pg*: pigment granule. *st*: cleavage stalk. *vm*: vitelline membrane.

which appears hyaline after the ejection of the cortical granules on fertilization (MOTOMURA '41 a) (see Fig. 1).

Judgement of the presence or the absence of a color becomes extremely doubtful, especially when the color becomes very faint. The author's criterion may be more dependable. At any rate, it is important to remember that both MOTOMURA's and the author's criteria are negative characteristics depending on the absence of some character.

RESULTS

1) *Oxalated- and citrated-Ca-free solutions*

In order to guard against possible out-diffusion of calcium ions and against the danger of injuring the cells, Na-oxalate and Na-citrate of the same tonicity as the natural medium were added to a Ca-free solution to the extent of 10 volume per cent. The Ca-free solution used was the chloride mixture without SO_4 ions. The fertilization membrane was removed by shaking (after MOTOMURA) and the eggs were washed in a tall cylindrical vessel by settling and changing the supernatant, care being taken to insure a complete mixing yet to avoid any violent stirring.

In spite of the above precautions, the new surface appeared in every case. In the citrated solution, the blastomeres fell widely apart immediately after cleavage (drawing closer later) and the extent of the *de novo* surface appeared to be less than in the straight Ca-free solution. In order to make this conclusion more objective, the total surface area of the blastomere and the area of the *de novo* part were computed from camera lucida drawings by a method given in the foregoing paper (DAN and ONO '54). The results are given in Table 1.

In this table, the percentage increases of the total surface at the 2-cell and 4-cell stages against the pre-cleavage spherical condition are around 130% and a little above 160%, respectively (3rd and 5th columns). Since the theoretical figures for the increase of surface following the division of an original sphere into 2 or 4 equally sized spheres are 126.1% and 158.8%, the consistently higher observed figures indicate that the blastomeres are not strictly spherical. This tendency is particularly clear in the 4-cell stage, in the oxalated and citrated solutions.

If such increases are to be taken care of by the development of *de novo* surface, the expected percentages of the *de novo* surface on the blastomeres are 26.1/126.1=20.7% and 58.8/158.8=37.0%. This indicates that in the chloride mixture, the observed *de novo* surfaces are larger than the theoretical values, in the oxalated solution, the *de novo* values at the 4-cell stage approach that level, while in the citrated solution, the observed figures are lower in both 2- and 4-cell stages.

In the table, the data are arranged according to the extent of *de nove* surface formed at the 2-cell stage. If the corresponding figures for the 4-cell stage (the last column) are compared, although the correlation is not so clear for the chloride mixture, it becomes more evident as one proceeds from the oxalated to the citrated solutions. Therefore, it is possible to state that the extent of the

Table 1.

	1 cell	2 cell		4 cell	
		% increase of total surface	% of *de novo* on blastomere	% increase of total surface	% of *de novo* on blastomere
Chloride mixture	100.0	131.7	43.4	165.5	55.8
		122.5	30.4	158.2	57.8
		132.4	27.6	162.8	33.9
		125.1	27.3		
		129.9	26.1		
		124.0	24.8	181.4	56.6
		128.0	24.5		
		142.4	22.4		
		126.8	21.6	143.1	42.9
		137.8	21.3	169.7	45.5
		142.6	19.3	172.7	47.0
		122.8	14.0	140.3	
		126.0	13.8	155.6	52.5
Ave.	100.0	130.2	24.3	161.0	58.5
Oxalated Ca-free	100.0	130.8	35.3	154.3	66.4
		141.2	31.1	162.8	52.3
		132.6	27.8	175.1	51.2
		133.2	27.3	163.9	51.7
		115.4	25.6	163.2	16.7
		129.1	19.9	162.9	9.4
		131.7	13.3	201.4	35.2
		129.7	12.9		
Ave.	100.0	130.5	24.2	169.1	40.7
Citrated Ca-free	100.0	130.2	24.8	171.3	30.4
		128.3	23.1	164.9	51.2
		136.1	18.1	165.5	46.4
		127.9	15.8	171.3	30.4
		130.3	15.7	137.0	20.1
		131.7	12.6	197.7	31.5
		130.2	10.6	163.1	17.5
		128.3	4.4	163.6	15.3
		131.4	1.5	169.5	13.2
Ave.	100.0	130.5	14.7	167.1	28.4

de novo formation decreases in the order, chloride mixture > oxalated Ca-free > citrated Ca-free, although the *de novo* formation is never suppressed entirely.

2) *Change in the ionic composition of the medium*

The above difference in the degree of *de novo* formation in the three solutions suggests that calcium ions may not be the sole factor controlling it, which, in turn, leads to the suspicion that the difference between the conclusions reached by MOTOMURA and the author might be due to the sulphate ions which were included in MOTOMURA's Ca-free and omitted from the author's.

Several solutions were prepared, each containing different anions or urea, and their effects on the formation of the *de novo* surface were tested. The compositions of the solutions used are given in Table 2.

A single batch of eggs was divided into several groups, and each group

Table 2. Constitution of various Ca-free solutions tested.
(The given figures are grams per 1 liter)

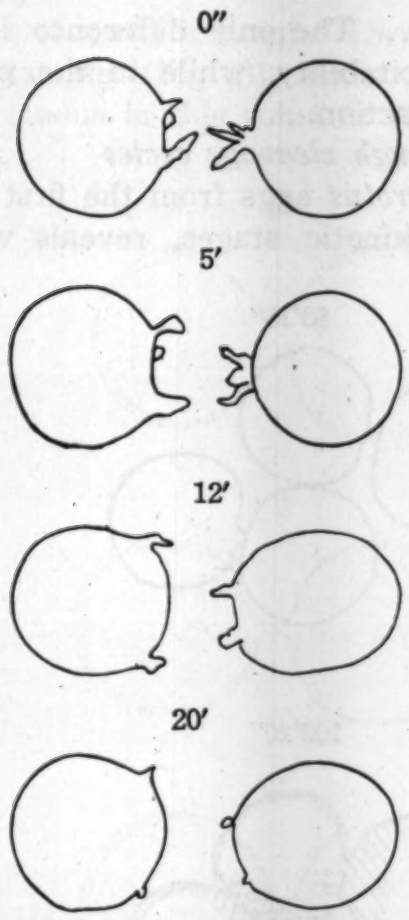
	NaCl	$NaHCO_3$	$NaNO_3$	$NaNO_2$	NaSCN	KCl	KBr	KI	$MgSO_4 \cdot 7H_2O$	$MgCl_2 \cdot 6H_2O$	Urea
I	24.0	0.5				0.7				14.6	
II	26.9	0.5				0.7			11.9		
III	26.1	0.5				3.5			11.0		
IV	21.2	0.5	4.08			0.7				14.6	
V	21.2	0.5		3.3		0.7				14.6	
VI	21.2	0.5			3.9	0.7				14.6	
VII	21.8	0.5					5.7			14.6	
VIII	21.8							8.0		14.6	
IX	22.6	0.5				0.7				14.6	2.9

was washed by the solution to be tested. Judgement was made either by a general survey of the cultures or by comparing camera lucida drawings of representative eggs taken at random from them. The range of variation of the results is fairly wide, at least to the same extent as that given in Table 1. Yet, the author feels it possible to ascribe characteristic effects to various substances.

In urea-containing Ca-free solution (IX), the new surface is usually very clear. But occasionally this mixture causes the blastomeres to become amoeboid. In this condition, the entire surface appears uniform and it is impossible to detect the new surface. This is presumably due to the disturbance caused by the amoeboid motion, for when amoeboid motion occurs in only one of the two blastomeres, it is always possible to recognize the new surface in its quiescent partner.

In SO_4-containing Ca-free solution (II, III), the extent of the new formation is much less, but the new part is definitely identifiable. In one batch of eggs, the extra-granular zone was relatively thin from the beginning and when the eggs divided in SO_4-containing Ca-free solution (II), no new surface was detectable and the blastomeres became amoeboid just as in MOTOMURA's description. However, in this case, after the completion of the cleavage process, amoeboid processes on the cell surface moved out of the furrow in the same way as do kaolin particles, indicating that essentially the same surface movement is occurring, although the structural difference is obscured by the amoeboid motion (Fig. 2).

Solution V, containing NO_2 ions, suppresses the new formation more than



do those containing SO_4 ions. It is slightly toxic so that the pace of cleavage is delayed by 50 minutes in reaching the 4-cell stage.

$NaNO_3$ (IV) is so toxic that no division is possible.

NaSCN (VI) is relatively toxic. If the eggs are put into this mixture soon after fertilization, no cleavage takes place. If they are transferred immediately before the first cleavage, the eggs can undergo two divisions, reaching the 4-cell stage. In this solution, the surface structure remains uniform through two cleavage cycles, including the interkinetic stages. In other words, the *de novo* surface is completely suppressed.

Fig. 2. The movement of the furrow surface of one special batch of *Hemicentrotus* eggs in a SO_4-containing Ca-free solution, as indicated by amoeboid processes.

Disregarding the concentration differences among the above substances for the time being, if the effects of these substances are arranged in the order in which they allow the development of the new surface, the following anion series is obtained:

Cl, oxalate > I, urea > Br, SO_4 > citrate, NO_2 > SCN.

The above series reminds one of the HOFMEISTER lyotropic series more than anything else, in spite of the slightly anomalous positions of SO_4 and citrate. In the HOFMEISTER series, the left hand members of the series cause shrinkage and the right hand members enhance the swelling of neutral gelatine gel. It is well known that the cortical layer of fertilized sea urchin eggs with which we are here concerned is a gel and this gel is probably negatively charged, like gelatine gel in the neutral reaction (*cf.* DAN '47). If this supposition be true, the whole problem of the new surface will need a fundamental revision.

The effect of SCN adds weight to this possibility. As already mentioned, this ion abolishes the formation of the new surface completely. However, if SCN-treated eggs are transferred from SCN solution to the straight Ca-free solution at the middle of the interkinetic stage, the new surface appears within a few minutes; while if eggs which have acquired the new surface in other solutions are put into SCN solution, the already formed new surface remains unaffected. This situation is strikingly similar to the effect of anions on the excitability of frog muscles studied by SCHWARZ ('07). Excitability which is lost in the left hand members of the lyotropic series comes back in solutions of the right hand members while after the loss of excitability in solutions of the right hand members, transferrence of the muscle to solutions of the left

members of the series cannot restore excitability. The only difference is that in the muscle, gelification seems to abolish excitability, while in the present material, gelification enhances the cortical contraction.

3) *Continuous observation of the cortex through cleavage cycles*

Detailed continuous observation of *Hemicentrotus* eggs from the first to the second or third cleavage, including the interkinetic stages, reveals various

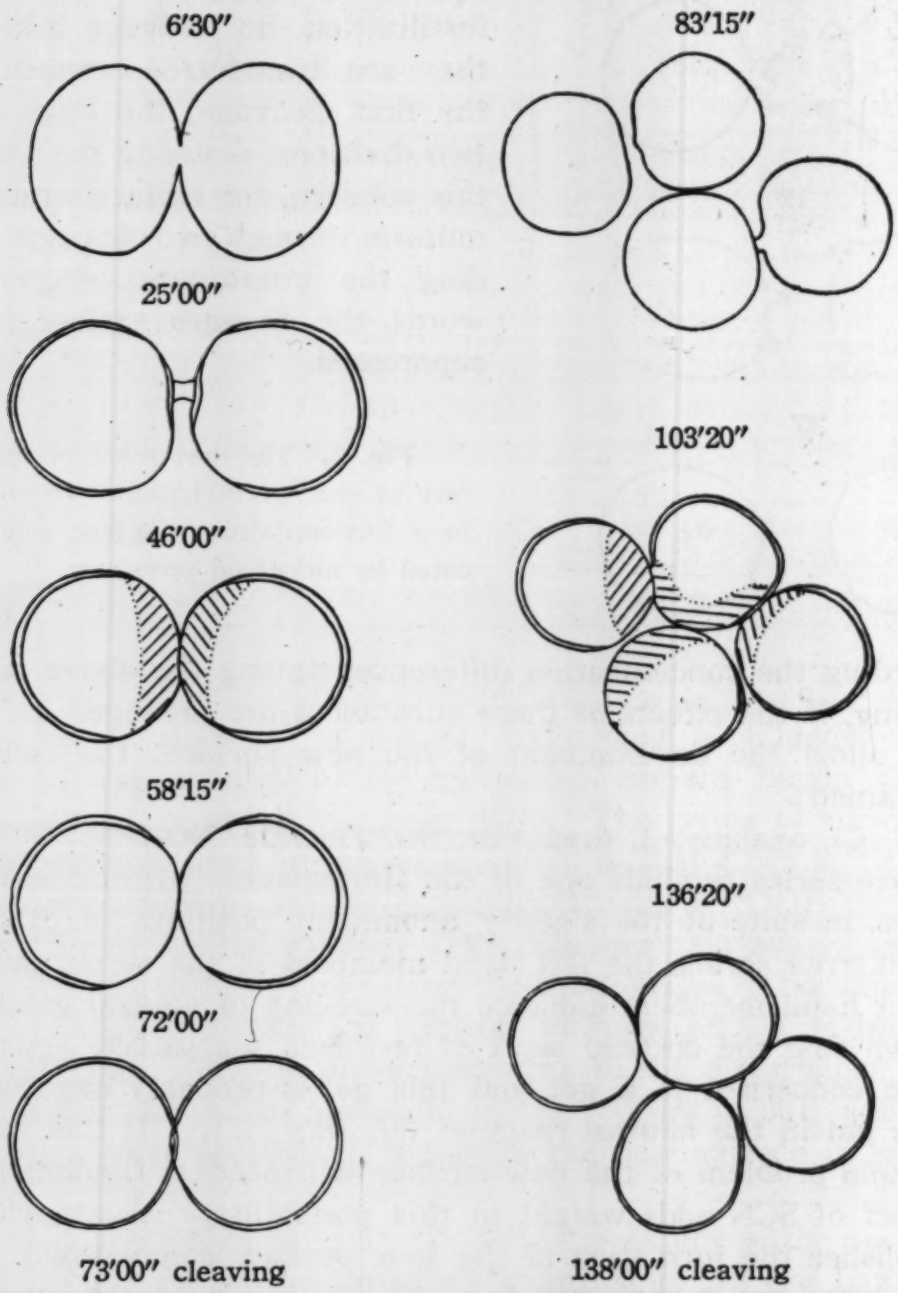


Fig. 3. Cleavage in oxalated Ca-free solution at 13° C. 1) cleavage being completed 2) accumulation of the orange pigment and the extra-granular zone at the base of the stalk 3) recession of the extra-granular zone and the orange pigment of the furrow side. After the recession of the pigment, the furrow surface assumes a grey color, which is shown by shading. 4) and 5) return of the orange pigment and the extra-granular zone — obliteration of the "new" surface. 6) completion of the 2nd cleavage 7) the shift of the cortical material for the 2nd interkinetic period 8) return of the cortex to a uniform condition — obliteration of the "new" surface.

points of great interest. The case of oxalated Ca-free medium will be given as an example (Fig. 3), since various phases of the cortical changes are more conspicuous in this solution than in others (Fig. 4).

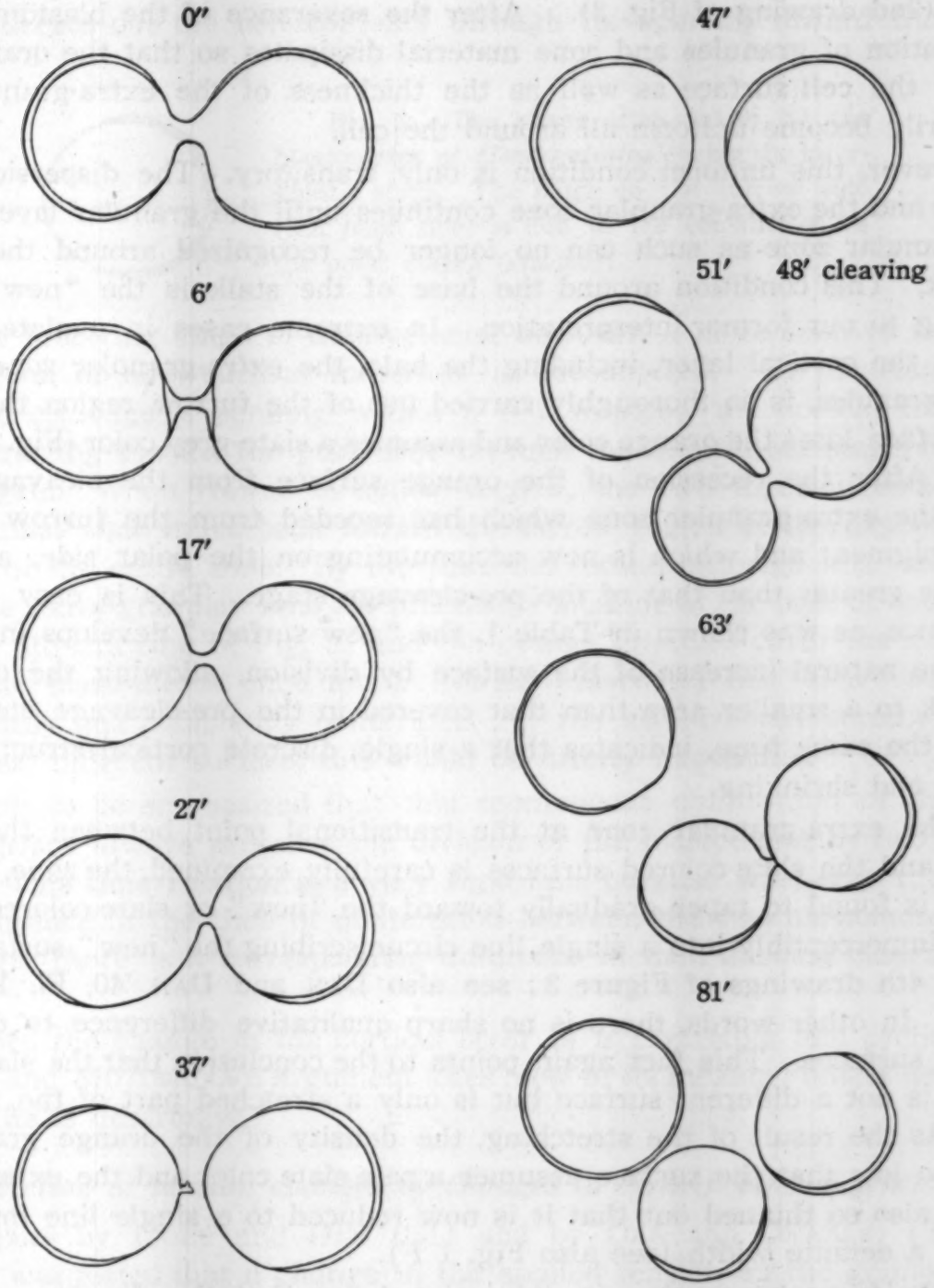


Fig. 4. Cleavage in citrated Ca-free solution. The first drawing represents an egg at 3 hours 20 minutes after fertilization at 12.1° C. 1) cleavage is approaching completion 2) accumulation of the pigment and the extra-granular zone at the base of the stalk 3), 4) and 5) apperance of the "new" surface. Note the bulge of the furrow sides of the cells. 6) in the right blastomere, the cortex is re-distributing itself uniformly once more. 7), 8) and 9) right blastomere: the normal sequence of the cortical movement during the 2nd interkinetic period; left blastomere: correlation between the re-distribution of the cortical material and the onset of a cleavage. Unless the cortex becomes uniform, cleavage does not take place (compare 6) and 9)).

Immediately after the completion of cleavage, the orange granules are accumulating around the connecting stalk. This fact has already been reported by both MOTOMURA and the author. However, on close observation, it is possible to find that the extra-granular zone of this part is also thicker than in other regions (2nd drawing of Fig. 3). After the severance of the blastomeres, the accumulation of granules and zone material dissipates so that the orange coloration of the cell surface as well as the thickness of the extra-granular zone temporarily become uniform all around the cell.

However, this uniform condition is only transitory. The dispersion of the granules and the extra-granular zone continues until the granular layer and the extra-granular zone as such can no longer be recognized around the base of the stalk. This condition around the base of the stalk is the "new surface" according to our former interpretation. In extreme cases in oxalated Ca-free solution, the cortical layer, including the halo, the extra-granular zone and the orange granules, is so thoroughly carried out of the furrow region that the *de novo* surface loses the orange color and assumes a slate-grey color (Fig. 3, shaded parts). After the recession of the orange surface from the cleavage furrow region, the extra-granular zone which has receded from the furrow with the orange pigment and which is now accumulating on the polar side, acquires a thickness greater than that of the pre-cleavage stage. This is easy to understand since, as was shown in Table 1, the "new surface" develops in excess to cover the natural increase of the surface by division, allowing the old cortex to shrink to a smaller area than that covered in the pre-cleavage stage. This fact, at the same time, indicates that a single, discrete cortical structure is expanding and shrinking.

If the extra-granular zone at the transitional point between the orange-colored and the slate-colored surfaces is carefully examined, the zone, in optical section, is found to taper gradually toward the "new" or slate-colored side and change imperceptibly into a single line circumscribing the "new" surface. (The 3rd and 4th drawings of Figure 3; see also DAN and DAN '40, Pl. 1, Figs. *D* and *E*). In other words, there is no sharp qualitative difference to demarkate the two surfaces. This fact again points to the conclusion that the slate-colored surface is not a different surface but is only a stretched part of the same surface. As the result of the stretching, the density of the orange granules becomes so low that the surface assumes a pale slate color and the extra-granular zone is also so thinned out that it is now reduced to a single line instead of a zone of a definite width (see also Fig. 1 *F*).

In the foregoing section, it was pointed out that the development of the "new surface" is affected to various degrees by different kinds of ions in the medium, and the effects of the ions seem to fall into the category of the HOFMEISTER lyotropic series. If the slate-colored surface were really a new surface of a different quality, it is rather difficult to understand why such a fundamental activity is so easily affected by the ions in the surrounding medium; while if the whole situation is considered to be a simple contraction of the cortex toward the polar side, the consistency of the contracting gel will easily be affected by the ionic composition of the medium according to the lyotropic series.

This idea offers an easy explanation concerning the way in which the blastomeres deviate from the spherical condition during the interkinetic stage. Typically they assume the shape illustrated in Figure 5. When the polar cortex is contracting, the cortical layer will act like the rubber bulb of a pipette so that it squeezes out the cell contents through its opening toward the furrow side.

Fig. 5. The shape often taken by the blastomeres of *Hemicentrotus* during the interkinetic stage. The bulge on the furrow side (left hand side) is due to the contraction of the polar cortex (shaded).

In the following stage of development, however, a more decisive fact negating the idea of new surface formation is encountered. In the stage when the second cleavage is pending within a few minutes, the cortex, which has been contracting toward the polar side through the interkinetic stage, begins to expand again. When viewed in optical section, the two tapering ends of the extra-granular zone move back toward the furrow region at the expense of the thickness of the entire zone. By the time the second cleavage sets in, the two ends of the extra-granular zone are practically meeting at the base of the former stalk (the 5th drawing of Fig. 3) and the orange granules cover the entire surface of the blastomeres once more. Stated otherwise, the "new" surface is spontaneously and completely obliterated. If the slate-colored surface were a qualitatively different surface, this would be utterly impossible.

It needs to be emphasized that this spontaneous obliteration of the slate-colored surface always precedes the division of the blastomeres by two or three minutes. This time relation is a very rigid one, because whenever there is a slight difference in the time of obliteration between the two blastomeres of the 2-cell stage, there is a corresponding difference in their ensuing cleavage time (Fig. 4).

In short, the change in the cortical layer is a reversible and cyclical phenomenon, and MOTOMURA's argument loses most of its meaning unless he defines the stage he is describing.

4) *Detection of tension changes by changes in cortical birefringence*

In papers by INOUÉ and DAN ('51) and by DAN and OKAZAKI ('51), the possibility was tested that a change in the applied tension on the gelified structure of cells might bring about a change in the birefringence of that structure. The absolute magnitude of the original birefringence is usually increased on stretching the structure, while it decreases on compression. Therefore, if the various changes in the cortex described above are due to a contraction of one part and a passive stretching of the adjoining part, it would be interesting to study the changes in the double refraction of these regions.

As is well known (RUNNSTRÖM '28; RUNNSTRÖM, MONNÉ and BROMAN '44), the cortical layer of sea urchin eggs has a negative birefringence and the hyaline layer has a positive birefringence in the direction of the tangent. Moreover,

MONROY and MONTALENTI ('47) found that such birefringence of sea urchin eggs undergoes cyclical changes, going through a maximum at the beginning of cleavage and passing through a minimum during the interphase.

Restricting our description to the cortical birefringence in the absence of the hyaline layer in a Ca-free medium, *Hemicentrotus* eggs behave in much the same way, and the strength of the double refraction fluctuates continuously. Unlike the photographs of MONROY and MONTALENTI ('47) or of MITCHISON and SWANN ('52), since the author used a mica compensator for sensitization, he had only two quadrants (opposite each other) to compare. At the very end of the spherical stage immediately before the first division, the cortical birefringence is found to reach a maximum. When the cleavage furrow appears, the shallow initial furrows are lined with surfaces having a weaker birefringence than the surface on the diametrically opposite side (which is not strictly in the polar region). This conclusion is somewhat different from those of the previous workers mentioned above. The furrow at this stage is in the shrinkage phase, as revealed by the kaolin experiment. From this stage on, the general birefringence begins to fade rather rapidly. Still it is possible to say that the surface of a deeper, well advanced furrow, which is definitely found to be in the expansion phase by the kaolin experiment, has a stronger birefringence than the polar surface. By this time the polar surface is shrinking, according to the kaolin experiments. Therefore, when considered with the result of the kaolin experiment (DAN, YANAGITA and SUGIYAMA '37), these findings bear out the correlation between the changes in membrane tension and in birefringence.

After the cleavage is completed, when the slate-colored surface appears, it is surrounded by a very fine but well-defined, brilliant line suggesting that the cortex of this part is highly stretched and thinned out, while the polar surface has a very faint birefringence. At the end of the interkinesis, when the orange-colored polar cortex begins to come back toward the furrow, the polar surface acquires a definitely stronger birefringence than that of the furrow region. When the orange surface covers the entire cell, the latter is uniformly surrounded by a very brilliant contour, and the birefringence reaches the second maximum prior to the second cleavage.

Translating the birefringence data into a picture of the tension changes, a conclusion similar to that arrived at before can be reached. Shortly after the completion of cleavage, the surface around the base of the stalk seems to become weakened; as a result, the cortex contracts at the expense of this weakened membrane. How much the original cortex will shrink depends upon the relative values of the membrane tension between the slate-colored and the orange-colored regions. If the cortical gel is softened by the lower members of the anions of the lyotropic series, it is understandable that its tendency to contract will be reduced and the development of the slate-colored area will be slight or lacking (SCN ions).

However, as the next cleavage draws near, this weak layer seems to develop the same strength as the rest of the surface, which results in pulling back the contracted membrane. The fact that the membrane tension is increased by this action is also shown by the rounding up of the blastomeres in preparat-

ion for each division. However, it is admitted that why and how the cortex reconstitutes itself before each division cannot be explained at present. At any rate, it is a suggestive fact that the cortical movement and the cortical birefringence go through cyclic changes in close parallel.

Discussion

In this section, some analyses will be made in an attempt to understand why this phenomenon was misinterpreted as the formation of a *de novo* surface. Motomura opened this series of studies with the observation that the apposed blastomere surfaces of the 2-cell stage of *Hemicentrotus* dividing in sea water are devoid of the orange granules. He confirmed this fact further by separating the blastomeres by shaking. However, when eggs are washed in a Ca-free medium and are allowed to cleave in it, the pigment-free surface does not appear ('35). In the next year, he expressed the opinion that the appearance of *de novo* surface (lacking the orange granules) in excess of the pre-existing surface causes the advance of the cleavage furrow. If this were true, how the eggs could ever cleave in the Ca-free medium is another matter. Since 1942, he has announced the discovery of a row of cytoplasmic vacuoles in fixed sections of the eggs of *Temnopleurus hardwickii* and suggested that the fusion of such vacuoles would result in furrow formation (Motomura '50).

On the other hand, the conclusion arrived at from the kaolin experiments turned out to be different, *i.e.*, the "new surface" does not appear until the cleavage is complete because if the apposed surfaces were entirely *de novo*, no kaolin particles should be carried into the cleavage furrow. Consequently it can be said that the formation of the new surface itself cannot be a part of the division mechanism.

The reason which led the author to decide that this post-cleavage phenomenon must be the formation of a new surface was derived from the shape of the blastomeres at interkinesis in normal *sea water*. It is admitted that here is a jump in the argument. As is well known, in normal sea water where the hyaline layer is preserved, the blastomeres come close together during interkinesis and take a hemispherical shape. Under this circumstance, the surface increase could attain a figure as high as 50% of the original surface area. The author thought that in order to account for such an increase, *de novo* formation must be resorted to. However, it is possible to imagine two ways in which an increase of surface area may occur. Either the surface may expand as the result of an increase in surface material, or else some external force may act to stretch the existing surface. Not being able to find evidence indicating the latter situation, the author adopted the first alternative.

Recently, however, Dan and Ono ('52) became aware that the latter situation does hold true in sea urchin eggs cleaving in *sea water*. We have noticed that the surfaces of fertilized sea urchin eggs are covered with fine processes which run between the egg surface and the overlying hyaline layer. As a result, the surface layer of the fertilized eggs is tightly bound to the hyaline layer by the mediation of these processes. Some experiments were reported

in the cited paper which indicate this fixation, and the name "attachment fibers" was proposed for these processes. After washing the eggs with a Ca-free medium, the attachment fibers remain on the egg surface, forming the halo referred to in previous sections of this paper, which corresponds to HERBST's Strahlensaum ('00), although HERBST thought this was the hyaline layer itself which had changed its form following the removal of calcium ions.

If such a connection exists, it is easy to imagine that when an egg divides in the natural medium with the hyaline layer intact, hardly any movement of the surface material is possible, since the hyaline layer is a relatively inelastic structure. Since no slipping is allowed between the hyaline layer and the cell surface, the only place where the two layers diverge is the furrow region where the cell surface sinks as the furrow whereas the hyaline layer remains outside, bridging over the furrow. Yet the connection between the two layers is not severed as the attachment fibers are simply stretched (see DAN and ONO '52 Fig.), indicating that the advancing furrow is being pulled back toward the hyaline layer. Because of this pull, when the blastomeres "relax" during interkinesis, the furrow sides are pulled back toward the hyaline layer. This, in turn, exerts a pull on the apposed surfaces and makes them expand on the equatorial plane and thereby imparts to the blastomeres a hemispherical shape. In a previous paper (DAN, DAN and YANAGITA '38 p. 530), the author pointed out the fallacy involved in the commonly used expression, "the blastomeres come closer together" to describe the situation in interkinesis, and said that it must be replaced by another expression, "the hemispherical shape is due to a spreading of the contact surfaces," which is still true even now.

From what has been said above, it can be inferred that when an egg cleaves without the hyaline layer in a Ca-free medium, the cell surface has a higher degree of freedom for movement. When one part of the surface is strained, the strain can be liquidated by a shift in position as well as by expansion of the adjacent parts. Moreover, being free from the restraining force imposed upon them by the inelastic extraneous layer, the blastomeres can assume a more nearly spherical shape. All these factors work together to reduce the pulling strain which is normally exerted on the furrow side, in sea water. Consequently, the furrow surface is not forced by an external force to reach an equilibrium condition (if the interkinetic condition is considered as an inactive equilibrium condition) but is allowed to reach that state gradually by its own power. Therefore, except for these superficial differences resulting from whether the hyaline layer is present or absent, there is no reason to think that the cortical behavior changes under the two conditions, as MOTOMURA claims. The difference lies only in the magnitude of the strain and in the time lag in reaching the steady states.

Concluding, all MOTOMURA's observations were correct, but he was somewhat overhasty in concluding that the advancing cleavage furrow is covered by a qualitatively different surface. What the author observed was also correct, but he was erroneous in confusing the conditions prevailing in a Ca-free medium with those in sea water.

The author expresses his indebtedness to Miss K. OKAZAKI, who made the birefringence

observations which were reported in this paper.

Summary

1) The former conclusion that a new surface is formed on the cleavage furrow region of sea urchin blastomeres is revised.

2) The "new" surface which is characterised by the absence of the extra-granular zone and the orange-colored granules is actually a locally stretched part of the pre-existing surface.

3) This stretching is brought about by the contraction of the rest of the cortex toward the polar region, as evidenced by a thickening of the extra-granular zone and an increase in density of the orange granules.

4) The consistency of the contracting cortical gel is affected by various anions in the surrounding medium, according to the Hofmeister lyotropic series.

5) As a result of this study, it is now possible to explain the differences between the cortical movement as it occurs under the hyaline layer and that which is found in a Ca-free medium, without the hyaline layer.

Literature

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THE CORTICAL MOVEMENT IN *ARBACIA PUNCTULATA* EGGS THROUGH CLEAVAGE CYCLES*

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In previous papers, the movement of the cortex of *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* was reported (DAN and DAN '40; DAN '54). Previous conclusions were reached by observations of three different kinds, *i.e.*, the movement of kaolin particles attached to the cell surface, the change in the thickness of the optical section of the extra-granular zone of the cortex, and finally the change in distribution of the orange-colored granules on the surface. Data obtained by the kaolin method measure regional expansion or shrinkage of the surface. Consequently, in interpreting the result, these separate data have to be integrated into a composite picture. Although observations of the change in the thickness of the extra-granular zone and of the distribution of the orange-colored granules are better in this respect, judgement is reliable only when the changes are extreme, since the criteria are not delicate enough to give information on changes of intermediate degrees.

As is well known, the eggs of the Woods Hole sea urchin, *Arbacia punctulata*, have conspicuous red pigment granules. These pigment granules are not only larger and easier to discern than the orange granules of *Hemicentrotus*, but they make superb landmarks to indicate the movement of the cortex, since, after fertilization, they are imbedded firmly in the cortex in a single layer of uniform density (DAN and DAN '40, Plate II, C).

METHOD

In the present study, the cortical movement of *Arbacia* eggs in a Ca-free medium was followed through four division cycles to the 16-cell stage, by watching the distribution of the red granules. To trace the change in pigmentation, microphotographs were taken, using Ansco color film. The microscope diaphragm was fully opened, minimizing the formation of ordinary microscopic figures (defraction images), in order to obtain a pure color effect. This situation can be realized by looking at the micromeres of the 16-cell stage in Figure 27 Pl. 5, which are hardly visible in the photograph because of the lack of

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pigment granules in these blastomeres. Observations were made on cells sealed in deep depression slides to avoid mechanical disturbances.

Results

In the uncleaved condition (Fig. 1, Plate 4), the red pigment granules are distributed uniformly at the periphery of the cell. During the cleavage process (Fig. 2), the granules accumulate around the cleavage furrow, which means that the furrow surface is shrinking in area. This accumulation persists through the cleavage process (Fig. 3), even until the early interkinetic stage (Fig. 4). While the granules are accumulating around the cleavage furrow, their distribution on the polar side becomes sparser, showing that the surface of this region is being stretched (see Dan and Ono '54).

Shortly after the completion of cleavage, the granule accumulation around the cleavage stalk disperses and the distribution temporarily becomes uniform around the cell (Fig. 5). This dispersion of the granules, however, continues so that, in the next moment, the red color begins to fade on the furrow sides (Fig. 6). Besides the shift of the pigment granules, the second characteristic feature of this stage is the bulging of the blastomere contours toward the furrow side. The author thinks that this form change and also the pigment shift are due to the contraction of the major part of the cortical layer toward the polar side and a consequent stretching and thinning of the furrow surface (Dan '54). The third characteristic of this stage is a reduction in the distance between the two blastomeres, which were clearly separated immediately after the completion of the preceding cleavage (compare Fig. 5 with Figures 6 and 7). Although the blastomeres are not literally touching in this particular case. quite frequently they come lightly in contact with each other. In order to see this phenomenon, it is absolutely necessary to keep the eggs sealed in a depression slide. As long as this precaution is taken, the same situation obtains in *Hemicentrotus pulcherrimus* (see Figures *D* and *E* of Dan and Dan '40) and *Mespilia globulus*.

In 1941, Motomura objected to the author's conclusion on this ground, saying that the fact that the blastomeres are in contact with each other in our illustrations (Dan and Dan '40, Plate 4, Figures *D* and *E*) was a sign of calcium contamination. This objection is not valid since not only this coming together of the blastomeres invariably happens simultaneously with the recession of the pigment from the furrow side but also the blastomeres spontaneously move apart again when the next cleavage approaches (Fig. 11)

During the latter half of each interkinetic stage, the one-sided distribution of the pigment granules is gradually levelled off (Figures 7, 8, 9, 10 and 11). As the red pigment returns to the furrow side, the two blastomeres go apart again as was pointed out above. In the egg photographed, the pigment return was not perfect (Fig. 11) and a small area which has less pigment is left on the furrow side. This is carried over to the second cleavage (Fig. 12).

The correlation between the return of the pigment and the ensuing cleavage is also very rigid. In other words, if the pigment return fails to occur, the cells will never divide. If there is a difference in the time of redistribution between

the two blastomeres, there is a corresponding difference in the cleavage time. What can be said at present is that the cortex seems to reconstitute itself before each division and this is a prerequisite of the coming cell division.

In the second division and its interkinetic stages, essentially the same process is repeated. Thus during the second division, the pigment accumulates along the furrow surface (Figures 12 and 13), and then disperses during the interkinesis (Figures 14 and 15). As a result, the pigment-carrying cortex is pushed to the outer edges of the four blastomeres, leaving the inner apposing sides poor in pigment. In Fig. 15, the pigment shift is clear in two blastomeres, but not so clear in the remaining ones. This is because the two blastomeres rotated in slightly different directions when the cells elongated for division, since the second cleavage spindles were not horizontal. The direction of the blastomeres on the right hand side is perfect, as their furrow regions and the polar regions are in good view, while in the other two, the polar regions are turned to the lower side. However, it is not hard to obtain typically symmetrical distribution, which looks exactly like the extra-granular zone of *Hemicentrotus* eggs as illustrated in the previous paper (DAN '54). At any rate, this distribution is such that the pigment granules are shifted to the outer sides of the blastomeres, while the inner sides are poor in pigment.

As the third division draws near, the pigment granules tend to take a uniform distribution for the third time (Fig. 16). (These cells began to cleave 2 minutes after Fig. 16 was taken). However, generally speaking, the redistribution of the pigment is not so complete at this time as it is at the end of the 2-cell stage. As a matter of fact, there is an increasing tendency for unequal distribution to persist as the division cycle advances, as if there occurs a shortage of cortical material as the total surface area of the blastomeres increases.

In Plate 5, another series of records is shown. Fig. 17 is a picture taken at the completion of the second cleavage. Fig. 18 shows the stage of uniform pigmentation. Figures 19 and 20 show the middle of the interkinetic stage with the maximal shift of the pigment. Fig. 19 is a surface view of Fig. 20, indicating how uneven the distribution can be. Fig. 21 shows the last stage of the 4-cell stage with the return of the pigment layer to the furrow side and the spontaneous separation of sister blastomeres. Fig. 22 is the onset of the third cleavage.

Figure 23 shows the beginning of the 8-cell stage. The four blastomeres at the two ends would have been arranged on top of the inner four cells if the larva had been kept in normal sea water. Moreover, by an examination of later stages, it can be ascertained that the four blastomeres at the end make up the animal half of the larva, so that in normal sea water, the four end-cells would be resting on the central vegetal cells with the egg axis standing vertical to the substratum. Figs. 24 and 25 are pictures taken at the height of the pigment shifting. Again it is evident that the pigment granules are pushed to the peripheral parts of the larva. Fig. 26 is the very beginning of the fourth cleavage. It can be noticed that the return is less complete than in earlier cleavages. This is understandable because, as the number of blastomeres increases, it will become more difficult for the original cortex to expand to surround all the cells.

Fig. 27 is the 16-cell stage, in which 8 mesomeres, 4 macromeres and 4 micromeres are to be expected. But there is some ambiguity in the cleavage pattern of the present material. Starting from the inside, four cells out of five marked in the explanatory figure (Fig. *A*) as "*M*" must be macromeres and four out of five marked "*m*" should be micromeres, since it is known that the micromeres are always devoid of pigment in this species. About the mesomeres, two paris on both sides marked "Mes_1" and "Mes_2" are clearly mesomeres, since they have cleaved equally with a uniform distribution of the pigment. A pair designated as "Mes_3" on the right hand side are almost certainly mesomeres as they are of quasiequal sizes and pigmented, although not equally. Then the fourth pair of mesomeres must be one of the five unequal pairs marked "*M-m*" in the central group. The author thinks it may very likely be the $M_?$-$m_?$ pair. The upper left-hand cell in Fig. 26 must have shifted its position when this culture was agitated to bring all the cells to lie flat on the substratum. At any rate, this is clearly an abnormal division both from the size relation as well as from the pigment distribution. In other words, one pair of mesomeres have divided by mistake into a macromere and a micromere of the natural proportion.

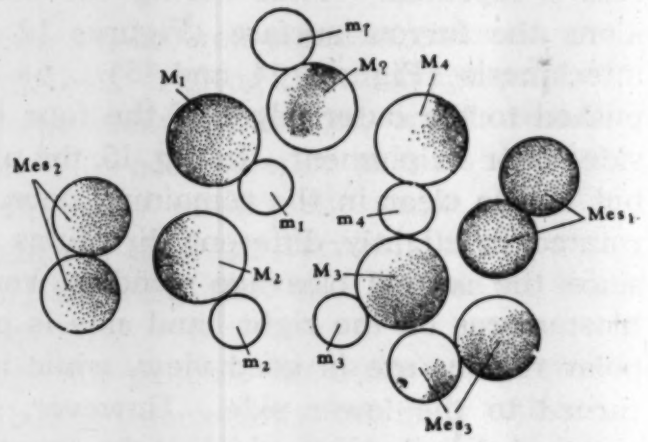

Fig. *A*

At present the cause of this abnormality is not known. However, it is extremely interesting that there seems to be a correlation between the unequalness of the cell size and that of the pigment distribution. It has been known for a long time in the normal embryology of this species, that the animal side cells of the 8-cell stage divide equally with an equal amount of the pigment, while on the vegetal side, the macromeres receive most of the pigment and the micromeres are colorless. Apparently this situation holds for abnormally produced unequal divisions as well.

Diluted Ca-free medium

In the preceding paper (DAN '54), it was reported that the addition of thiocyanide anions (SCN) suppresses the cortical movement of the interkinetic stage of *Hemicentrotus* eggs so that the polar shift of the cortical material which normally occurs is completely inhibited, and uniform distribution of the orange granules is maintained through the interkinetic stages. During the process of the second cleavage, a pigment concentration is seen around the cleavage furrow, which disperses only to a uniform condition again.

During the present study, it was found that Ca-free solution diluted to the extent of 80% suppresses the cortical movement in a similar fashion without impairing the division activity (Figures 28–37, Plate 6). Considering the fact that, in *Hemicentrotus*, the contraction of the cortex toward the polar side was suppressed by various anions apparently by the order of the lyotropic series, the

effect of the dilute Ca-free solution may also be working through the softening of the cortical gel.

The larvae of Plate 6 went through the first division 3 minutes before Fig. 28 was taken. Starting from the onset of the division, Figures 29–32 were taken at intervals of 6, 11 and 7 minutes respectively. In spite of the fact that the observation well covered the interkinetic period, no sign of the polar migration of the pigment is detectable. The same is true with the 4-cell stage. Figures 34–37 were taken at intervals of 10, 9 and 7 minutes. Since observation was continued in between time, there is no possibility that the polar contraction of the cortex would have been missed if it had taken place.

There are two interesting correlations among these cortical changes. The first is that when the polar contraction of the cortex fails, the bulging of the furrow contour also fails and the blastomeres remain spherical through the interkinetic stages (see Figures 30 and 31 and compare with Pl. 4 Fig. 6). Previously it was argued that the bulging of the blastomere contours must be a result of the polar contraction of the cortex (see DAN '54). The present fact fits in with this explanation. The second point is a correlation between the pigment migration and the mutual attraction or repulsion of the blastomeres. In dilute Ca-free medium, the distance between newly cleaved blastomeres is usually wide. This distance decreases as the pigment accumulation at the base of the stalk disperses to a uniform condition (Figures 29-31). So far, the behavior is more or less like that in undiluted Ca-free solution. At the end of the interkinetic stage, however, because of the failure of the polar shift of the pigmented cortex, no return is possible. Parallel with this, the re-widening of the distance between the blastomeres at this stage cannot be seen (Figures 31–33). In Fig. 33, a slight difference in cleavage time will be noticed between two upper blastomeres. Through Figures 34 and 35, it can be seen that later happenings such as pigment shift and mutual movement of the blastomeres also did not occur simultaneously.

From these considerations, it can be inferred that the change in distance between the blastomeres is also somehow connected with the pigment migration, as it is with the bulging of the furrow contour. It seems as though the dispersing movement of the cortex in the earlier half of the resting stage pulls in the stalk substance, thus making the blastomeres draw each other in, while the returning movement pushes it out again, thus pushing the blastomeres away from each other.

Division of polyspermic eggs

In *Arbacia*, polyspermy can easily be caused by increasing the magnesium ions in the medium (CLARK '36). When the eggs divide irregularly by polyspermy, regardless of whether the egg divides simultaneously into three or four or more cells, each furrow is always bridged over by a cleavage stalk. Therefore, when an egg divides into three cells, there is a triradiate stalk at the center. If three blastomeres are arranged in an L, producing two separate furrows, there are two separate stalks, the middle blastomere having two stalks at two separate points of the cell. When an egg divides into four, there is a

cross-shaped stalk (Fig. 38). The author has shown (DAN and DAN '47) that this kind of stalk is not a simple crossing of two stalks but it is actually conversion of two or more V's.

At any rate, in the division of polyspermic eggs, pigment-poor areas invariably appear at the bases of the stalks during the interkinetic stage (Fig. 39). In the L-shaped division, the effect is particularly striking because, on account of the abnormal positions of the two stalks formed, the middle blastomere develops two pigment-poor parts, each corresponding to one stalk. From this it can be said that the appearance of the pigment-free areas is directly correlated with the presence of the stalk.

DISCUSSION

MOTOMURA was the first to discover in *Hemicentrotus* (*Strongylocentrotus*) that the orange granules which surround the periphery of the uncleaved eggs are found, in the blastula stage, still on the outer surface of the larvae (MOTOMURA '35). This is an extremely interesting finding. On the other hand, the author has recently reported on the attachment of the surface layer of fertilized sea urchin eggs to the overlying hyaline layer (DAN and ONO '52). This attachment helps to explain Motomura's finding, because such an attachment prevents the cortical material from migrating into the interior of the blastula.

However, what the present study reveals is that the peripheral position of the cortex is more than an accidental physical effect of the extraneous coat, since the blastomeres themselves possess an intrinsic capacity to bring about the same effect. So here we find a beautiful example of harmony between intrinsic and extrinsic factors in the developmental process.

At present, it is impossible to know exactly what significance this phenomenon has in an embryological sense. But in this connection, particularly interesting is the behavior of the micromeres. These mesenchyme cells are destined to migrate into the segmentation cavity earlier than other elements of the larvae and they are denuded of the cortical material already at the time of their formation.

SUMMARY

1) The cortical movement of *Arbacia punctulata* eggs in a Ca-free medium was followed by recording the migration of the red pigment granules by color photography.

2) As was found in *Hemicentrotus pulcherrimus* by other methods, the furrow cortex shrinks and the polar surface expands during the division process of *Arbacia* egg. Following the completion of cleavage, the pigment which has accumulated around the cleavage stalk disperses and by the middle of interkinesis, it is found concentrated in the opposite, polar region. Toward the end of interkinesis, it returns again, to be uniformly distributed around the cell surface.

3) Rather rigid correlations are noticed between pigment granule (cortical) movement and cytokinetic activity. With the dispersion of the pigment from the stalk base, a) the newly formed blastomeres come closer together. With

the polar migration of the pigment, b) the blastomere contours bulge toward the furrow. With the return of the pigment to a uniform condition at the end of the interkinesis, c) the blastomeres go away from each other and they go into the next division. These relations are diagrammatically represented in Fig. B.

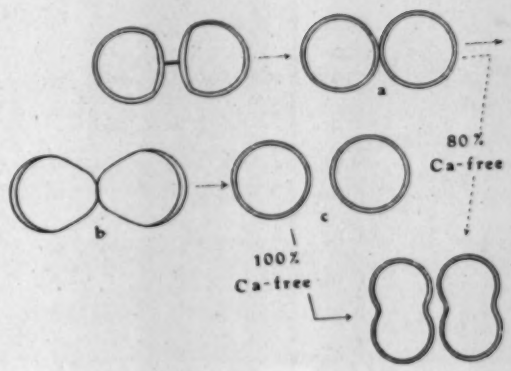

Fig. *B*

4) In a diluted Ca-free medium, although the furrow-side accumulation of the pigment disperses to a uniform condition, the polar concentration of the pigment fails to occur. Corresponding to these features, although the blastomeres come closer together (a), neither the bulging of the cell contour (b) nor the later going apart of the blastomeres occurs (c). However, this medium does not prevent the ensuing division because a uniform pigment distribution is secured through interkinesis.

5) In polyspermic eggs which cleave irregularly, a pigment-poor area is formed corresponding to each cleavage stalk, no matter how abnormal its position is.

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Explanations of Figures

PLATE 4. *Arbacia punctulata* eggs in Ca-free medium 24.1° C.

Fig. 1. 43 minutes 00 seconds after insemination.
Fig. 2. 49 m. 00 s.
Fig. 3. 50 m. 25 s.
Fig. 4. 51 m. 45 s.
Fig. 5. 53 m. 15 s.
Fig. 6. 55 m. 30 s.
Fig. 7. 60 m. 30 s.
Fig. 8. 65 m. 25 s.
Fig. 9. 68 m. 25 s.
Fig. 10. 72 m. 30 s.
Fig. 11. 74 m. 00 s.
Fig. 12. 77 m. 00 s.
Fig. 13. 78 m. 50 s.
Fig. 14. 81 m. 30 s.
Fig. 15. 88 m. 40 s.
Fig. 16. 99 m. 15 s.
This egg began to deivide at 101 m. 15 s.

PLATE 5. *Arbacia punctulata* eggs in Ca-free medium (continued) 22.6° C. (Original is in color).

Fig. 17. 74 minutes 15 seconds after insemination.
Fig. 18. 77 m. 15 s.
Fig. 19. 86 m. 00 s. surface view.
Fig. 20. 87 m. 15 s.
Fig. 21. 93 m. 15 s.
Fig. 22. 97 m. 00 s.
Fig. 23. 101 m. 30 s.
Fig. 24. 107 m. 20 s. surface view.
Fig. 25. 108 m. 30 s.
Fig. 26. 122 m. 50 s.
Fig. 27. 133 m. 50 s.

PLATE 6. *Arbacia punctulata* eggs in 81.5% Ca-free medium 21.8° C. (Original is in color).

Fig. 28. 99 minutes 00 seconds after insemination. Two eggs are shown.
Fig. 29. 109 m. 15 s.
Fig. 30. 115 m. 10 a.
Fig. 31. 126 m. 31 s.
Fgi. 32. 133 m. 25 s.
The second division began at 134 m. 40 s.
Fig. 33. 137 m. 55 s.
Fig. 34. 143 m. 35 s.
Fig. 35. 153 m. 00 s.
Fig. 36. 162 m, 45 s.
Fgi. 37. 169 m. 40 s.
The third division started at 174 m. 30 s.

Division of polyspermic larva. 75% isotonic $MaCl_2$+25% sea water. 21.9° C.
Fig. 38. 76 minutes 10 seconds after insemination.
Fig. 39. 88 m. 55 s.

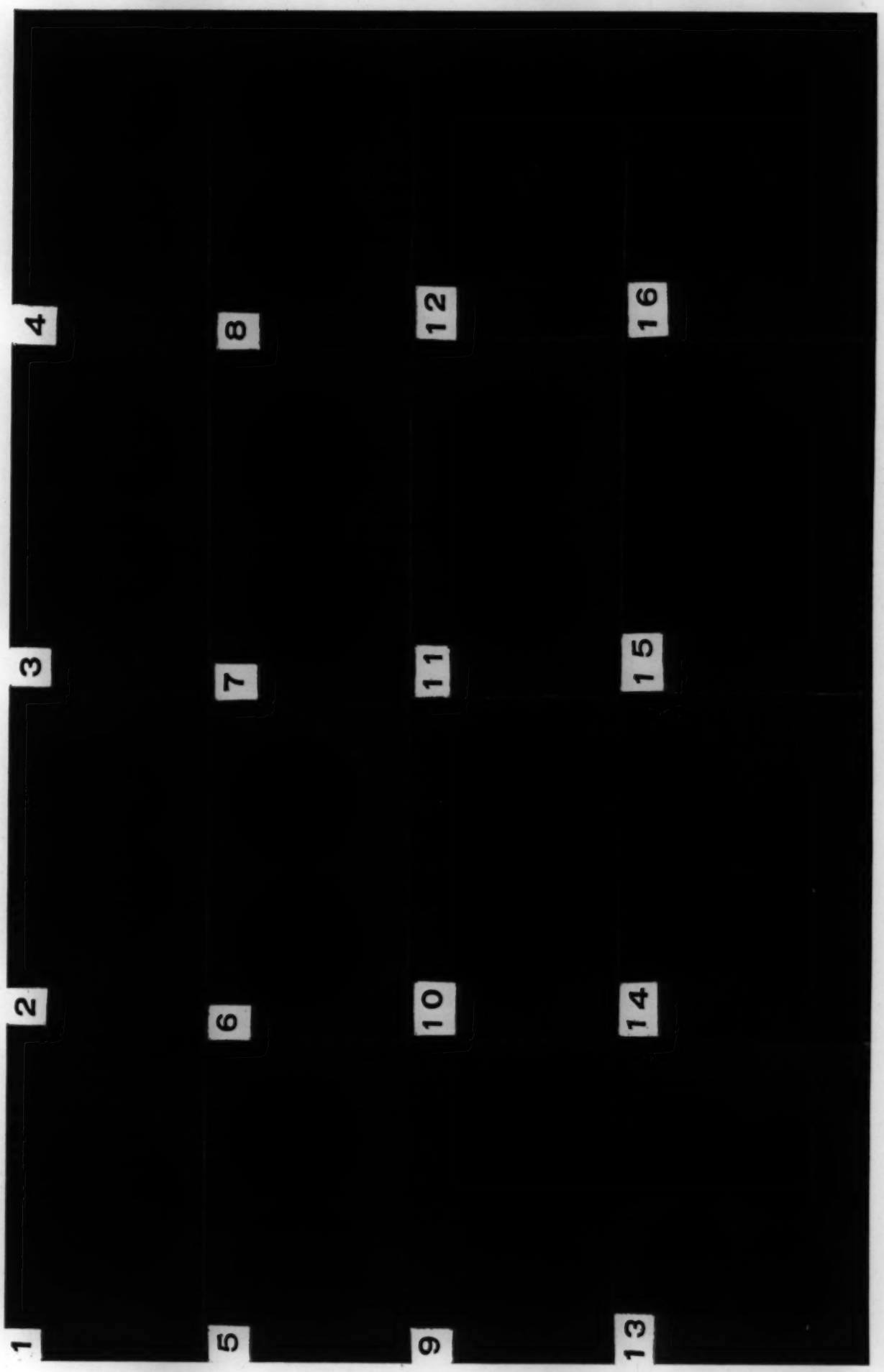
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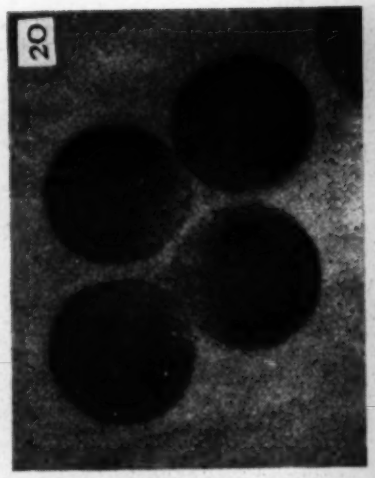
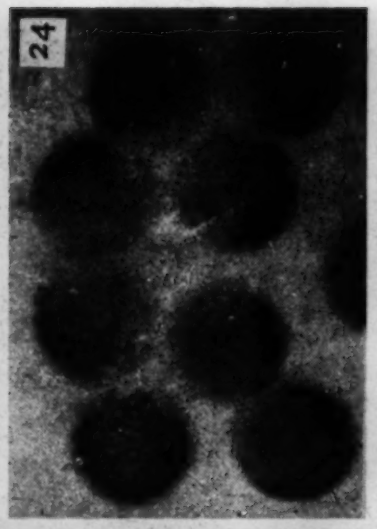
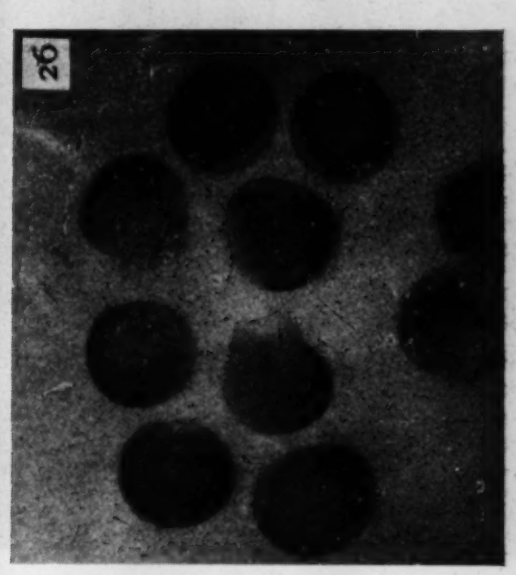
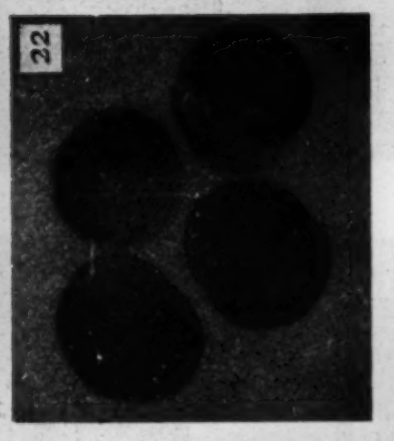
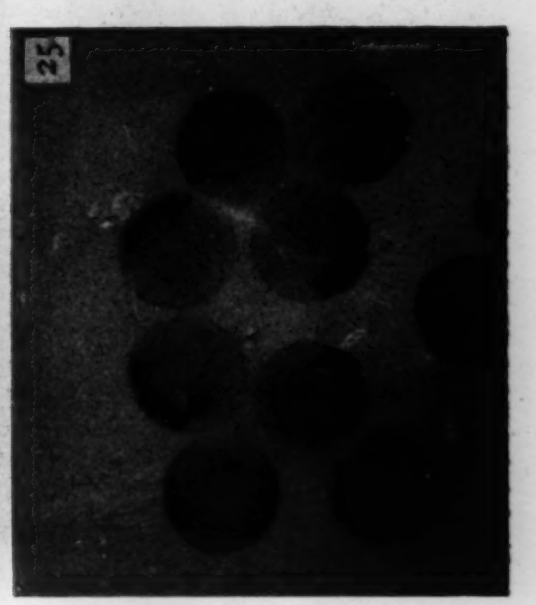
PLATE 5.

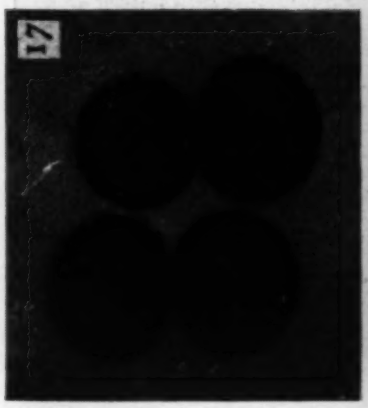


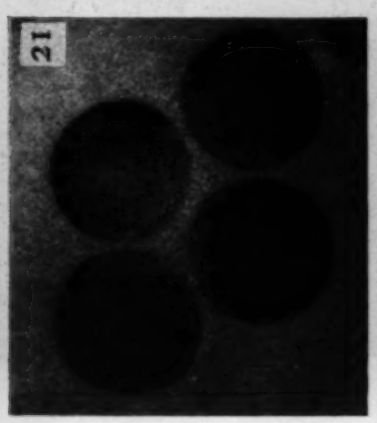










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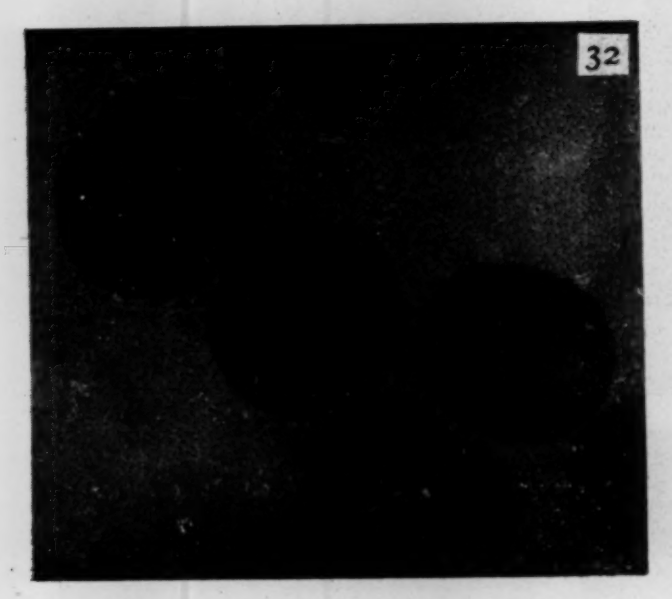
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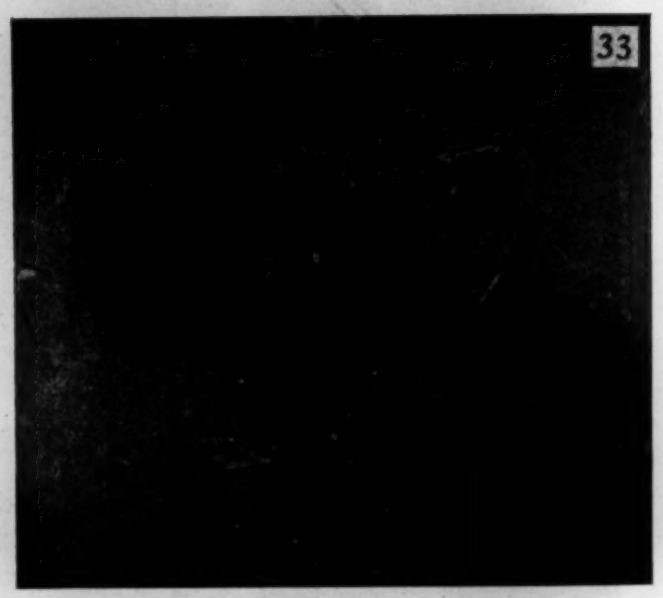
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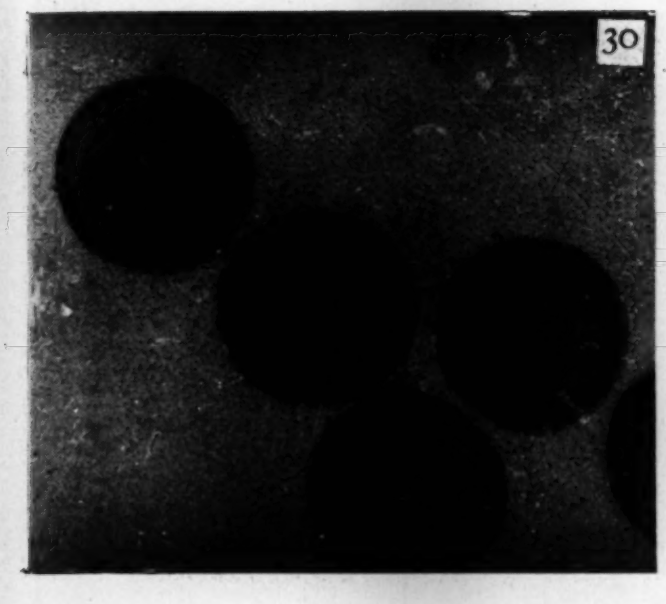
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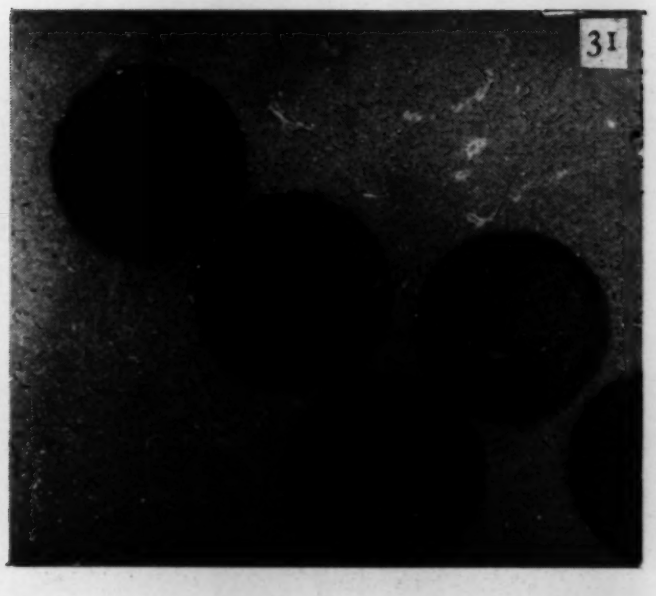
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